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(54) Title: ANTIGEN PRESENTING SYSTEM AND METHODS FOR ACTIVATION OF T-CELLS

(57) Abstract

The present invention relates to synthetic antigen-presenting matrices, their methods of making and their methods of use. One such matrix is cells that have been transfected to produce MHC antigen-presenting molecules and assisting molecules such as co-stimulatory molecules. The matrices can be used to activate CD8+ T-cells to produce cytokines and become cytotoxic.

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ANTIGEN PRESENTING SYSTEM AND
METHODS FOR ACTIVATION OF T-CELLS

This invention was made with the support of the
Government of the United States of America, and the
5   Government of the United States of America has certain
rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of
U.S. patent application Serial No. 08/400,338 filed
10   March 8, 1995 and having the same title.

TECHNICAL FIELD

The present invention relates to materials and
methods of activating T-cells with specificity for
particular antigenic peptides, the use of activated
15   T-cells in vivo for the treatment of a variety of disease
conditions, and compositions appropriate for these uses.

BACKGROUND

The efficiency with which the immune system
cures or protects individuals from infectious disease has
20   always been interest to scientists, as it has been
believed that it might be possible to activate the immune
system to combat types of diseases. Such diseases
include cancer, hepatitis and infectious disease in
immunosuppressed patients. While various procedures
25   involving the use of antibodies have been applied in
those types of disease, few if any successful attempts
using cytotoxic T-cells have been recorded.
Theoretically, cytotoxic T-cells would be the preferable
means of treating the types of disease noted above.
30   However, no procedures have been available to
specifically activate cytotoxic T-cells.

Cytotoxic T-cells, or CD8 cells (i.e., cells
expressing the molecule CD8) as they are presently known,
represent the main line of defense against viral
35   infections. CD8 lymphocytes specifically recognize and

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kill cells which are infected by a virus. Thus, the cost
of eliminating a viral infection is the accompanying loss
of the infected cells. The T-cell receptors on the
surface of CD8˙ cells cannot recognize foreign antigens
5   directly. In contrast to antibodies, antigen must first
be presented to the receptors.

The presentation of antigen to CD8˙ T-cells is
accomplished by major histocompatibility complex (MHC)
molecules of the Class I type. The major
10   histocompatibility complex (MHC) refers to a large
genetic locus encoding an extensive family of
glycoproteins which play an important role in the immune
response. The MHC genes, which are also referred to as
the HLA (human leucocyte antigen) complex, are located on
15   chromosome 6 in humans. The molecules encoded by MHC
genes are present on cell surfaces and are largely
responsible for recognition of tissue transplants as
"non-self". Thus, membrane-bound MHC molecules are
intimately involved in recognition of antigens by
20   T-cells.

MHC products are grouped into three major
classes, referred to as I, II, and III. T-cells that
serve mainly as helper cells express CD4 and primarily
interact with Class II molecules, whereas CD8-expressing
25   cells, which mostly represent cytotoxic effector cells,
interact with Class I molecules.

Class I molecules are membrane glycoproteins
with the ability to bind peptides derived primarily from
intracellular degradation of endogenous proteins.
30   Complexes of MHC molecules with peptides derived from
viral, bacterial and other foreign proteins comprise the
ligand that triggers the antigen responsiveness of
T-cells. In contrast, complexes of MHC molecules with
peptides derived from normal cellular products play a

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in the thymus. Class I molecules do not present entire, intact antigens; rather, they present peptide fragments thereof, "loaded" onto their "peptide binding groove".

5   For many years, immunologists have hoped to raise specific cytotoxic cells targeting viruses, retroviruses and cancer cells. While targeting against viral diseases in general may be accomplished *in vivo* by vaccination with live or attenuated vaccines, no similar success has been achieved with retroviruses or with

10   cancer cells. Moreover, the vaccine approach has not had the desired efficacy in immunosuppressed patients. At least one researcher has taken the rather non-specific approach of "boosting" existing CD8· cells by incubating them *in vitro* with IL-2, a growth factor for T-cells.

15   However, this protocol (known as LAK cell therapy) will only allow the expansion of those CD8· cells which are already activated. As the immune system is always active for one reason or another, most of the IL-2 stimulated cells will be irrelevant for the purpose of combatting

20   the disease. In fact, it has not been documented that this type of therapy activates <u>any</u> cells with the desired specificity. Thus, the benefits of LAK cell therapy are controversial at best, and the side effects are typically so severe that many studies have been discontinued.

25   Several novel molecules which appear to be involved in the peptide loading process have recently been identified. It has also been noted that Class I molecules without bound peptide (i.e., "empty" molecules) can be produced under certain restrictive circumstances.

30   These "empty" molecules are often unable to reach the cell surface, however, as Class I molecules without bound peptide are very thermolabile. Thus, the "empty" Class I molecules disassemble during their transport from the interior of the cell to the cell surface.

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The presentation of Class I MHC molecules bound
to peptide alone has generally ineffective in activating
CD8· cells. In nature, the CD8· cells are activated by
antigen-presenting cells which present not only a
5   peptide-bound Class I MHC molecule, but also a
costimulatory molecule. Such costimulatory molecules
include B7 which is now recognized to be two subgroups
designated as B7.1 and B7.2. It has also been found that
cell adhesion molecules such as integrins assist in this
10 process.

When the CD5· T-cell interacts with an
antigen-presenting cell having the peptide bound by a
Class I MHC and costimulatory molecule, the CD8· T-cell
is activated to proliferate and becomes an armed effector
15 T-cell. See, generally, Janeway and Travers,
Immunobiology, published by Current Biology Limited,
London (1994), incorporated by reference.

Accordingly, what is needed is a means to
activate T-cells so that they proliferate and become
20 cytotoxic. It would be useful if the activation could be
done in vitro and the activated cytotoxic T-cells
reintroduced into the patient. It would also be
desirable if the activation could be done by a synthetic
antigen-presenting cells comprised of a material such as
25 cells which not only presents the selected peptide, but
also presents other stimulatory factors which increase
the effectiveness of the activation.

It would also be advantageous if it was
possible to select the peptide so that substantially only
30 those CD8· cells cytotoxic to cells presenting that
peptide would be activated.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to a synthetic
antigen-presenting system for presenting an MHC molecule

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complexed to a peptide and an assisting molecule to a
T-cell to activate the T-cell.

In one embodiment, the system relates to a
synthetic antigen-presenting matrix having a support and
5   at least the extracellular portion of a Class I MHC
molecule capable of binding to a selected peptide
operably linked to the support. The matrix also includes
an assisting molecule operably linked to the support.
The assisting molecule acts on a receptor on the CD8⁺ T-
10   cell. The MHC and assisting molecules are present in
sufficient numbers to activate a population of T-cell
lymphocytes against the peptide when the peptide is bound
to the extracellular portion of the MHC molecule.

It has been found that an antigen-presenting
15   matrix having both an MHC molecule or a portion of a MHC
molecule together with an assisting molecule, provides a
synergistic reaction in activating T-cell lymphocytes
against the peptide. Examples of assisting molecules are
costimulatory molecules such as B7.1 and B7.2 or adhesion
20   molecules such as ICAM-1 and LFA-3. The extracellular
portion of such costimulatory molecules can also be used.
Another type of costimulatory molecule is one that reacts
with the CD28 molecule such as anti-CD28 antibodies or
functional portions thereof, e.g. Fab portions.
25   It has been found that a specifically effective
synergistic reaction results from an antigen-presenting
matrix having MHC molecules bound with a peptide, a
costimulatory molecule, and an adhesion molecule. In
particular, a highly effective synergistic generation of
30   cytotoxic T-cell activity results from the combination of
B7.1 and ICAM-1.

The support used for the matrix can take
several different forms. Examples for the support
include solid support such as metals or plastics, porous
35   materials such as resin or modified cellulose columns,

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microbeads, microtiter plates, red blood cells and
liposomes.

Another type of support is a cell fragment,
such as a cell membrane fragment or an entire cell. In
5   this embodiment, the matrix is actually cells which have
been transfected to present MHC molecules and assisting
molecules on the cell surface to create an antigen-
presenting cell (APC). This is done by producing a cell
line containing at least one expressible Class I MHC
10   nucleotide sequence for the MHC heavy chain, preferably a
cDNA sequence, operably linked to a first promoter and an
expressible β-2 microglobulin nucleotide sequence
operably linked to a second promoter. The MHC heavy
chain and the β-2 microglobulin associate together form
15   the MHC molecule which binds to the peptide. The MHC
protein binds with the antigenic peptide and presents it
on the surface of the cell. The cell also includes a
gene for a nucleotide sequence of an assisting molecule
operably linked to a third promoter. The assisting
20   molecule is also presented on the surface of the cell.
These molecules are presented on the surface of the cell
in sufficient numbers to activate a population of T-cell
lymphocytes against the peptide when the peptide is bound
to the complexes. Other molecules on the surface of a
25   cell or cell fragment such as carbohydrate moieties may
also provide some costimulation to the T-cells.

The cell line is synthetic in that at least one
of the genes described above is not naturally present in
the cells from which the cell line is derived. It is
30   preferable to use a poikilotherm cell line because MHC
molecules are thermolabile. A range of species are
useful for this purpose. See, for example, U.S. Patent
No. 5,314,813 to Petersen et al. which discusses numerous
species for this use and is incorporated by reference.

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It is preferred to use eukaryotic cells and insect cells in particular.

In one embodiment, it is particularly preferred to have at least two assisting molecules, one being a
5   costimulatory molecule and the other being an adhesion molecule. It has been found that this combination has a synergistic effect, giving even greater T-cell activation than either of the individual molecules combined. It has also been found to be advantageous and preferable to have
10   at least one of the transfected genes under control of an inducible promoter.

Using the present invention, it is possible to introduce the peptide to the cell while it is producing MHC molecules and allow the peptide to bind the MHC
15   molecules while they are still within the cell. Alternatively, the MHC molecules can be expressed as empty molecules on the cell surface and the peptide introduced to the cells after the molecules are expressed on the cell surface. In this latter procedure, the use
20   of poikilotherm cells is particularly advantageous because empty MHC molecules, those not yet complexed or bound with peptides, are thermolabile.

Class I MHC molecules have been expressed in insect cells such as *Drosophila melanogaster* (fruit fly)
25   cells. Since *Drosophila* does not have all the components of a mammalian immune system, the various proteins involved in the peptide loading machinery should be absent from such cells. The lack of peptide loading machinery allows the Class I molecules to be expressed as
30   empty molecules at the cell surface.

Another advantage of using insect cells such as the *Drosophila* system is that *Drosophila* cells prefer a temperature of 28 °C rather than 37 °C. This fact is very important, because empty Class I molecules are
35   thermolabile and tend to disintegrate at 37 °C. By

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incubating the Class I-expressing *Drosophila* cells with
peptides that can bind to the Class I molecule, it is
possible to get virtually every Class I molecule to
contain one and the same peptide. The cells are
5   accordingly very different from mammalian cells, where
the Class I molecules contain many different types of
peptides, most of which are derived from our own,
innocuous cellular proteins.

The present invention also relates to methods
10   for producing activated CD8· cells *in vitro*. One method
comprises contacting, *in vitro*, CD8· cells with one of
the antigen-presenting matrices described above for a
time period sufficient to activate, in an
antigen-specific manner, the CD8· cells. The method may
15   further comprise (1) separating the activated CD8· cells
from the antigen-presenting matrix; (2) suspending the
activated CD8· cells in an acceptable carrier or
excipient; and (3) administering the suspension to an
individual in need of treatment. The antigens may
20   comprise native or undegraded proteins or polypeptides,
or they may comprise antigenic polypeptides which have
been cleaved into peptide fragments comprising at least 8
amino acid residues prior to incubation with the human
Class I MHC molecules.
25   In another variation, the invention relates to
methods treating conditions in patients and specifically
killing target cells in a human patient. The method
comprises (1) obtaining a fluid sample containing resting
or naive CD8· cells from the patient; (2) contacting, *in*
30   *vitro*, the CD8· cells with an antigen-presenting matrix
for a time period sufficient to activate, in an
antigen-specific manner, the CD8· cells; and (3)
administering the activated CD8· cells to the patient.
For example, the use of tumor specific peptides allows

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cytotoxic activated CD8· T-cells. The invention also
relates to the method of treating a medical condition by
administration of an antigen-presenting matrix in a
suitable suspension. In various embodiments the
5   condition may comprise cancer, tumors, neoplasia, viral
or retroviral infection, autoimmune or autoimmune-type
conditions. In one embodiment, the method of
administering the matrix comprises intravenous injection.

BRIEF DESCRIPTION OF THE DRAWINGS

10   Figures 1-3 diagram the construction of
expression plasmids pRmHa-2 and pRmHa-3. In Figure 1,
pRmHa-2 construction is shown; in Figure 2, pRmHa-3
construction is shown; and in Figure 3, the pRmHa-3
vector is illustrated, showing the restriction,
15   polylinker, promoter, and polyadenylation sites, as well
as a site at which a nucleotide sequence may be inserted
for expression;

          Figures 4 and 5 show peptide-induced
thermostabilization of HLA B27 and HLA A2.1 expressed on
20   the surface of Drosophila cells by HIV peptides. The
mean fluorescence of each cell population is shown
plotted against the incubation conditions;

          Figure 6 illustrates data from an experiment
designed to determine whether insect cells can process
25   antigen and load it onto the Class I molecules, and
whether the latter can present either endogenously or
exogenously derived antigen to T-cells. Schneider 2
(SC2) or 3T3 cells transfected with $K^b/\beta2$ were incubated
with ovalbumin protein (OvPro) or ovalbumin peptide, OVA
30   24 (OvPep) in isotonic (Iso) or hypertonic (Hyp) media.
(Murine cell line BALB/3T3 is available from the ATCC
under accession number CCL 163.) After treatment, cells
were cocultured with the T-cell hybridoma B3/CD8. B3/CD8
is a T-cell hybridoma between B3 (Carbone, et al., J.
35   Exp. Med. 169: 603-12 (1989)), cytotoxic T-cell specific

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for ovalbumin peptide 253-276 presented by H-2K^h Class I
molecules, and CD8- bearing IL-2-secreting cell line.
Upon antigenic stimulation, B3/CD8 produces IL-2,
measured by ^{3}H-thymidine incorporation in IL-2-dependent
5   cell line CTLL (Gillis, et al., J. Immunol. 120: 2027
91978)). Thus, by measuring the amount of IL-2 produced,
one can assay for T-cell recognition. The supernatant
from the cocultures were analyzed for IL-2 by ^{3}H
thymidine incorporation by the IL-2-dependent cell line
10   CTLL (ATCC No. TIB 214). The amount of ^{3}H thymidine
incorporated is plotted against the initial cell
treatments;

    Figure 7 illustrates the expression of B7.1,
ICAM-1 and MHC on the surface of transfected Drosophila
15   (fly) cells according to the present invention;

    Figure 8 is a graph showing results of a
fluorescence-activated cell sorter experiment using
recombinant L^d mouse MHC linked to red blood cells;

    Figure 9 is a graph showing results of a
20   fluorescence-activated cell sorter experiment using
recombinant K^h mouse MHC linked to red blood cells;

    Figure 10 is a graph demonstrates binding of
recombinant K^h to microtiter plates by use of labeled
antibodies;

25   Figure 11 is a series of graphs showing the
results from fluorescence-activated cell sorter
experiments demonstrating the expression of CD69 and CD25
on CD8$^+$ 2C cells stimulated with transfected Drosophila
cells;

30   Figure 12 is a pair of bar graphs showing
IL-2-dependent proliferative responses of CD8$^+$ 2C cells
elicited by peptides presented by Drosophila cells
transfected with L^d only;

    Figure 13 is a graph showing the influence of

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CD8⁺ 2C cells elicited by peptides presented by
transfected *Drosophila* cells;

      Figure 14 is a pair of graphs showing the
influence of antigen-presenting cells dose on day 3
5   proliferative response and IL-2 production of CD8⁺ 2C
cells elicited by peptides presented by transfected
Drosophila cells;

      Figure 15 is a series of graphs showing the
influence of peptide concentration on the proliferative
10   response of CD8⁺ 2C cells elicited by *Drosophila* cells
transfected with Lᵈ.B7, Lᵈ.ICAM and Lᵈ.B7.ICAM;

      Figure 16 is a series of graphs showing the
kinetics of the proliferative response of CD8⁺ and CD8⁻
2C cells elicited by *Drosophila* cells transfected with
15   Lᵈ, Lᵈ.B7, Lᵈ.ICAM and Lᵈ.B7.ICAM plus QL9 peptide;

      Figure 17 is a series of graphs showing CTL
activity of CD8⁺ 2C cells stimulated by *Drosophila* cells
transfected with Lᵈ.B7, Lᵈ.B7.ICAM or Lᵈ.ICAM antigen-
presenting cells plus QL9 peptide (10 μM) in the absence
20   of exogenous cytokines;

      Figure 18 is a pair of graphs showing CTL
activity of CD8⁺ 2C cells stimulated by *Drosophila* cells
transfected with Lᵈ.ICAM antigen-presenting cells plus
QL9 peptide (10 μM) in the absence (left) or presence
25   (right) of exogenous IL-2 (20u/ml);

      Figure 19 is a pair of graphs showing the
proliferative response of normal (non-transgenic) CD8⁺
T-cells to peptides presented by transfected *Drosophila*
cells (left panel) and the response elicited by graded
30   doses of N B6 and 2C B6 CD8⁺ cells cultured with 5 x 10ᵈ
P10.D2 (Lᵈ) spleen cells (2000 cGy) in the absence of
peptides for 3 days without the addition of exogenous
cytokines (right panel);

      Figure 20 is a graph showing stimulated
35   mitogenesis of purified 2C+ T-cells cultured (50,000

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cells per well) in plates coated with immobilized
molecules with peptide QL9 (solid line = L^d and anti-CD28
antibody, broken line = L^d only);

     Figure 21 is a bar graph showing stimulated
5   mitogenesis of purified 2C+ T-cells at day 5 in culture,
with various indicated peptides, cultured in 96-well
plates coated with L^d and anti-CD28 antibody (hatched
bars = no IL-2, black bars = IL-2 added);

     Figure 22 is a graphical representation of the
10 results of cytofluorometric analysis of cells recovered
after 12 days of culture in plates coated with L^d and
anti-CD28 antibody and exposed to peptide QL9, stained
using the 2C T-cell receptor specific antibody 1B2 (M2 =
positive staining cells, M1 = negative staining cells);

15   Figure 23 is a graphical representation of the
results of cytofluorometric analysis of cells recovered
after 12 days of culture in plates coated with L^d and
anti-CD28 antibody and exposed to peptide p2Ca, stained
using the 2C T-cell receptor specific antibody 1B2 (M2 =
20 positive staining cells, M1 = negative staining cells);

     Figure 24 is a graphical representation of the
results of cytofluorometric analysis of cells recovered
after 12 days of culture in plates coated with L^d and
anti-CD28 antibody and exposed to peptide SL9, stained
25 using the 2C T-cell receptor specific antibody 1B2 (M2 =
positive staining cells, M1 = negative staining cells);

     Figure 25 is a graph showing cytolysis of
target cells by activated T-cells (solid line = peptide
QL9, broken line = control peptide LCMV);
30   Figure 26 is a graphical representation of the
cytotoxic lysis resulting from activation of human CD8
T-cells with antigen-presenting cells loaded with
influenza matrix peptide;

     Figure 27 is a graphical representation of the

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T-cells with antigen-presenting cells loaded with HIV-RT peptide; and

Figure 28 is a graphical representation of the cytotoxic lysis resulting from activation of human CD8· T-cells with antigen-presenting cells loaded with tyrosinase peptide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a synthetic antigen-presenting system which can be used to activate T-cell lymphocytes. The activated CD8· T-cells proliferate, produce cytokines, become cytotoxic or some combination of these results. In one preferred embodiment, the system activates cytotoxic CD8· cells which then proliferate and then are activated to seek out and destroy target cells. The present invention can be used to activate T-cells in vitro and the activated T-cells are then returned to the patient from which they were originally derived or may be used in vivo activation of T-cells.

The synthetic antigen-presenting system of the present invention has two major components. The first component is at least the extracellular portion of the Class I MHC molecule which is capable of binding to a selected peptide. The second major component is an assisting molecule which assists in the activation of T-cells. In each case, the extracellular portion of a larger molecule may be used but in certain embodiments, the entire molecules are used.

For ease of description, MHC molecules will be discussed generally, with the understanding that an extracellular portion of the MHC molecule may be used. The portion of the MHC molecule necessary for the present invention is the part which binds to the selected peptide and presents the peptide to the T-cell.

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The peptide is selected to activate the
appropriate T-cell, depending on the treatment to be
conducted.   For example, in the treatment of particular
cancers, certain antigenic peptides are presented on the
5   surface of the cancer cells which will react with
activated T-cells.   Thus, it is appropriate to use a
peptide selected to activate the appropriate T-cells that
will then bind with and destroy the cancer cells.
The present invention allows the MHC molecules
10   to be produced by cells with the peptide already
complexed with the MHC molecule or to produce empty MHC
molecules which do not yet have a peptide complexed with
them.   This latter embodiment is particularly useful
since it allows the peptide to be chosen after the MHC
15   molecules are prepared.
A Class I MHC molecule includes a heavy chain,
sometimes referred to as an alpha chain, and a β-2
microglobulin.   As discussed herein, the extracellular
portion of the Class I MHC molecule is made up of an
20   extracellular portion of an MHC heavy chain together with
the β-2 microglobulin.
In preparing the extracellular portions of MHC
to be linked to a support, soluble molecules are prepared
as discussed below.   These molecules generally lack the
25   transmembrane and cytoplasmic domain in the MHC molecule.
The assisting molecule helps facilitate the
activation of the T-cell when it is presented with a
peptide/MHC molecule complex.   The present invention
includes two major categories of assisting molecules.
30   The first category is composed of costimulatory molecules
such as B7.1 (previously known as B7 and also known as
CD80) and B7.2 (also known as CD86) which binds to CD28
on T-cells.   Other costimulatory molecules are anti-CD28
antibodies or the functional portions of such antibodies,

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The other major category of assisting molecules
of the present invention are adhesion molecules. These
include the various ICAM molecules, which include ICAM-1,
ICAM-2, ICAM-3 and LFA-3. It has been found that the
5   combination of a peptide bound to an MHC molecule used in
conjunction with one of these assisting molecules
activates the T-cells to an extent previously not seen.
An even greater synergistic reaction has been
achieved by using a peptide-bound MHC molecule in
10   conjunction with both a costimulatory molecule and an
adhesion molecule. This has been found to be
particularly effective in producing cytotoxic CD8⁺ cells.
In accordance with the present invention, the
MHC molecule and the assisting molecule are operably
15   linked to a support such that the MHC and assisting
molecules are present in sufficient numbers to activate a
population of T-cells lymphocytes against the peptide
when the peptide is bound to the extracellular portion of
the MHC molecule. The peptide can be bound to the MHC
20   molecule before or after the MHC molecule is linked to
the support.
The support can take on many different forms.
It can be a solid support such as a plastic or metal
material, it can be a porous material such as commonly
25   used in separation columns, it can be a liposome or red
blood cell, or it can even be a cell or cell fragment.
As discussed in more detail below, in the case where a
cell serves as a support, the MHC and assisting molecules
can be produced by the cell. The MHC molecule is then
30   linked to the cell by at least the transmembrane domain
if not also the cytoplasmic domain which would not be
present in a soluble form of MHC.
The extracellular portions of MHC molecule and
assisting molecule can be linked to a support by
35   providing an epitope which reacts to an antibody

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immobilized on the support. In addition, the MHC or
assisting molecules can be produced with or linked to
(His)ₓ which would react with nickel in forming part of
the support. Other means to immobilize or link MHC
5   molecules to a support are well known in the art.
        As discussed above, the support can be a cell
membrane or an entire cell. In such a case, an
eukaryotic cell line is modified to become a synthetic
antigen-presenting cell line for use with T-cell
10   lymphocytes. For ease of description, antigen-presenting
cells (APC) will also be called stimulator cells.
Because empty MHC molecules are thermolabile, it is
preferred that the cell culture be poikilotherm and
various cell lines are discussed in detail below.
15   A culture of cells is first established. The
culture is then transfected with an expressible Class I
MHC heavy chain gene which is operably linked to a
promoter. The gene is chosen so that it is capable of
expressing the Class I MHC heavy chain. The cell line is
20   also transfected with an expressible β-2 microglobulin
gene which is operably linked to a second promoter. The
gene is chosen such that it is capable of expressing β-2
microglobulin that forms MHC molecules with the MHC heavy
chain. In the case of soluble extracellular portions of
25   MHC molecules to be used with solid supports and the
like, a truncated MHC heavy chain gene is used as
discussed in more detail below.
        The culture is also transfected with an
expressible assisting molecule gene operably linked to a
30   third promoter. The assisting molecule gene is capable
of being expressed as an assisting molecule which
interacts with the molecule on the T-cell lymphocytes.
As discussed below, each of these genes can be
transfected using various methods, but the preferred

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The cell line transfected is chosen because it
lacks at least one of the genes being introduced. It has
been found that insect cells are advantageous not only
because they are poikilothermic, but because they lack
5   these genes and the mechanisms which would otherwise
produce MHC molecules bound to peptides. This allows for
greater control over the production of peptide-bound MHC
molecules, and the production of empty MHC molecules.
The MHC heavy chain is preferably from a different
10   species, more preferably, a homeotherm such as mammals
and, optimally, humans.

The preferred cell line is a stable
poikilotherm cell line that has the first promoter being
inducible to control the expression of the MHC heavy
15   chain. It is preferred that the cell assembles empty MHC
molecules and presents them on the cell surface so that
the peptides can be chosen as desired.

The resulting MHC molecules bind to the peptide
and are present in sufficient numbers with the assisting
20   molecules on the surface of the cell to activate a
population of T-cell lymphocytes against the peptide when
the peptide is bound to the MHC cells.

In a further embodiment, a second assisting
molecule gene is also transfected into the cell culture.
25   In this case, the first assisting molecule gene can be
for a costimulatory molecule and the second assisting
molecule gene can be for an adhesion molecule.

It is preferred that at least one of the genes
and, in particular, the MHC heavy chain gene be linked to
30   an inducible promoter. This allows control over the
production of MHC molecules so that they are only
produced at a time when the peptide of interest is
available and presented in the culture to react with the
produced MHC molecules. This minimizes undesirable MHC
35   molecule/peptide complexes.

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Where the cell line already produces one or
more of the desired molecules, it is only necessary to
transfect the culture with an expressible gene for the
gene which is lacking in the cells. For example, if the
5   cells already present the MHC molecules on their surface,
it is only necessary to transfect the culture with an
expressible gene for the assisting molecule.

The peptide can be introduced into the cell
culture at the time the cells are producing MHC
10   molecules. Through methods such as osmotic shock, the
peptides can be introduced in the cell and bind to the
produced MHC molecules. Alternatively, particularly in
the case poikilotherm cell lines, the MHC molecules will
be presented empty on the cell surface. The peptide can
15   then be added to the culture and bound to the MHC
molecules as desired.

After the cells are produced having MHC and
assisting molecules on their surfaces, they can be
lyophilized and the fragments of the cells used to
20   activate the population of T-cell lymphocytes.

Transfected cultures of cells can be used to
produced extracellular portions of MHC molecules and
assisting molecules. The use of extracellular portions
in conjunction with supports such as solid supports has
25   certain advantages of production. Where living cells are
used to provide a synthetic antigen-presenting cell, at
least three genes, two to produce the MHC molecule and
one for the assisting molecule must be introduced to the
cell. Often, additional genes such as for antibiotic
30   resistance are also transfected.

Where a solid support system is being used, one
cell line can produce the extracellular portions of MHC
molecules while another cell line produces the
extracellular portion of the assisting molecule. The MHC

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then be harvested from their respective cultures. The
molecules are then linked to an appropriate support in
sufficient numbers to activate a population of T-cell
lymphocytes against a peptide when the peptide is bound
5   to the extracellular portion of the MHC molecule. From a
production standpoint, two different cultures can be
used, but it is also possible to use the same culture,
however, requiring that the culture be transfected with
the additional gene for the extracellular portion of the
10   assisting molecule.
            A further modification of this embodiment is to
provide a third culture of cells which is transfected
with an expressible second assisting molecule gene. In
this example, the second culture of cells produces
15   extracellular portions of the costimulatory molecule
while the third culture of cells produce an extracellular
portion of an adhesion molecule. The adhesion molecule
portions are harvested and linked to the support.
            The present invention also relates to a method
20   for activating CD8˙ T-cells against a selected peptide.
The method relates to providing a cell line presenting
MHC molecules binding a peptide and assisting molecules
on their surfaces. Naive CD8˙ T-cells can be obtained by
removal from a patient to be treated. The cultured cells
25   are then contacted with the CD8˙ T-cells for a sufficient
period of time to activate the CD8˙ T-cell lymphocytes
resulting in proliferation and transforming the T-cells
into armed effector cells.
            The activated CD8˙ T-cells can then be
30   separated from the cell line and put into a suspension in
an acceptable carrier and administered to the patient.
An alternative method involves the use of the synthetic
antigen-presenting matrix to activate the CD8˙ cells.
            It is preferred that human genes are used and,
35   therefore, human molecule analogs are produced. As shown

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in prior U.S. Patent No. 5,314,813, murine systems
provide particularly useful models for testing the
operation of T-cell activation and demonstrate the
applicability of the process for human systems. See also
5   Sykulev et al., _Immunity_ 1: 15-22 (1994).

            1.   Human Class I MHC Molecules
            Class I MHC molecules comprise a heavy chain
and a β-microglobulin protein. A human Class I MHC heavy
chain of the present invention is selected from the group
10 comprising HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G,
and more preferably, from the group comprising HLA-A,
HLA-B, and HLA-C. The heavy chains are useful in either
soluble or insoluble form. In the soluble ("sol") form,
a stop codon is engineered into the nucleotide sequence
15 encoding the HLA molecule of choice preceding the
transmembrane domain.
            While it is possible to isolate nucleotide
sequences encoding human Class I MHC heavy chains from
known, established cell lines carrying the appropriate
20 variants -- e.g., transformed cell lines JY, BM92, WIN,
MOC, and MG -- it is more practical to synthesize the
nucleotide sequence from a portion of the gene via
polymerase chain reaction (PCR), using the appropriate
primers. This method has been successfully used to clone
25 full-length HLA cDNA; for example, the sequences for
HLA-A25, HLA-A2, HLA-B7, HLA-B57, HLA-B51, and HLA-B37
are deposited in the GenBank database under accession
nos. M32321, M32322, M32317, M32318, M32319 and M32320,
respectively. Known, partial and putative HLA amino acid
30 and nucleotide sequences, including the consensus
sequence, are published (see, e.g., Zemmour and Parham,
Immunogenetics 33: 310-320 (1991)), and cell lines
expressing HLA variants are known and generally available
as well, many from the American Type Culture Collection

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synthesize human Class I MHC-encoding nucleotide
sequences which may then be operatively linked to a
vector and used to transform an appropriate cell and
expressed therein.

5   Particularly preferred methods for producing
the Class I MHC heavy chain, β-2 microglobulin proteins
and assisting molecules of the present invention rely on
the use of preselected oligonucleotides as primers in a
polymerase chain reaction (PCR) to form PCR reaction
10   products as described herein. Gene preparation is
typically accomplished by primer extension, preferably by
primer extension in a polymerase chain reaction (PCR)
format.

          If the genes are to be produced by (PCR)
15   amplification, two primers, i.e., a PCR primer pair, must
be used for each coding strand of nucleic acid to be
amplified. (For the sake of simplicity, synthesis of an
exemplary HLA heavy chain variant sequence will be
discussed, but it is expressly to be understood that the
20   PCR amplification method described is equally applicable
to the synthesis of β-2 microglobulin, costimulatory
molecules, adhesion molecules, and all HLA variants,
including those whose complete sequences are presently
unknown.)

25   The first primer becomes part of the antisense
(minus or complementary) strand and hybridizes to a
nucleotide sequence conserved among HLA (plus or coding)
strands. To produce coding DNA homologs, first primers
are therefore chosen to hybridize to (i.e. be
30   complementary to) conserved regions within the MHC genes,
preferably, the consensus sequence or similar, conserved
regions within each HLA group -- i.e., consensus
sequences within HLA-A, HLA-B, HLA-C, and the
less-polymorphic groups, HLA-E, -F, and -G.

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Second primers become part of the coding (plus) strand and hybridize to a nucleotide sequence conserved among minus strands. To produce the HLA-coding DNA homologs, second primers are therefore chosen to
5   hybridize with a conserved nucleotide sequence at the 5' end of the HLA-coding gene such as in that area coding for the leader or first framework region. In the amplification of the coding DNA homologs the conserved 5' nucleotide sequence of the second primer can be
10   complementary to a sequence exogenously added using terminal deoxynucleotidyl transferase as described by Loh et al., Science 243: 217-220 (1989). One or both of the first and second primers can contain a nucleotide sequence defining an endonuclease recognition site. The
15   site can be heterologous to the immunoglobulin gene being amplified and typically appears at or near the 5' end of the primer.

The high turn over rate of the RNA polymerase amplifies the starting polynucleotide as has been
20   described by Chamberlin et al., The Enzymes, ed. P. Boyer, PP. 87-108, Academic Press, New York (1982). Another advantage of T7 RNA polymerase is that mutations can be introduced into the polynucleotide synthesis by replacing a portion of cDNA with one or more mutagenic
25   oligodeoxynucleotides (polynucleotides) and transcribing the partially-mismatched template directly as has been previously described by Joyce et al., Nuc. Acid Res. 17: 711-722 (1989). Amplification systems based on transcription have been described by Gingeras et al., in
30   PCR Protocols, A Guide to Methods and Applications, pp 245-252, Academic Press, Inc., San Diego, CA (1990).

PCR amplification methods are described in detail in U.S. Patent Nos. 4,683,192, 4,683,202, 4,800,159, and 4,965,188, and at least in several texts

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for DNA Amplification", H. Erlich, ed., Stockton Press,
New York (1989); and "PCR Protocols: A Guide to Methods
and Applications", Innis et al., eds., Academic Press,
San Diego, California (1990). Various preferred methods
5   and primers used herein are described hereinafter and are
also described in Nilsson, et al., Cell 58: 707 (1989),
Ennis, et al., PNAS USA 87: 2833-7 (1990), and Zemmour,
et al., Immunogenetics 33: 310-20 (1991), for example.
In particular, it is preferred to design primers from
10   comparison of 5' and 3' untranslated regions of HLA
alleles (e.g., -A, -B, -C, -E, -F, or -G alleles), with
selection of conserved sequences. Restriction sites may
also be incorporated into the 5' and 3' primers to enable
the amplification products to be subcloned into
15   sequencing or expression vectors. It may also be helpful
to place a 4-base spacer sequence proximal to the
restriction site to improve the efficiency of cutting
amplification products with enzymes.
          The following primers are preferred for
20   amplification of HLA-A, -B, -C, -E, -F, and -G cDNA,
preferably in separate reactions. Resulting cDNAs may
then be cloned and sequenced as described herein. These
primers are appropriate for use in amplifying all known
and presently unknown types of HLA.
25   HLA A
               5' primer: 5' CC ACC ATG GCC GTC ATG GCG CCC
     3'   (SEQ ID NO 1)
               3' primer: 5' GG TCA CAC TTT ACA AGC TCT GAG
     3'
30   (SEQ ID NO 2)
     HLA B
               5' primer: 5' CC ACC ATG CTG GTC ATG GCG CCC
     3'
               (SEQ ID NO 3)

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          3' primer: 5' GG ACT CGA TGT GAG AGA CAC ATC
3'

          (SEQ ID NO 4)

  HLA C

5   **5' primer:**   5' CC ACC ATG CGG GTC ATG GCG CCC
3'

          (SEQ ID NO 5)

          3' primer: 5' GG TCA GGC TTT ACA AGC GAT GAG
3'

10   (SEQ ID NO 6)

  HLA E

          5' primer: 5' CC ACC ATG CGG GTA GAT GCC CTC C
3'

          (SEQ ID NO 7)

15   **3' primer:**   5' GG TTA CAA GCT GTG AGA CTC AGA
3'

          (SEQ ID NO 8)

  HLA F

          5' primer: 5' CC ACC ATG GCG CCC CGA AGC CTC
20 3'

          (SEQ ID NO 9)

          3' primer: 5' GG TCA CAC TTT ATT AGC TGT GAG A
3'

          (SEQ ID NO 10)

25 HLA G

          5' primer: 5' CC ACC ATG GCG CCC CGA ACC CTC
3'

          (SEQ ID NO 11)

          3' primer: 5' GG TCA CAA TTT ACA AGC CGA GAG
30 3'

          (SEQ ID NO 12)

          In preferred embodiments only one pair of first
and second primers is used per amplification reaction.

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plurality of different amplifications, each using a
plurality of different primer pairs, are then combined.
However, the present invention also relates to DNA
homolog production via co-amplification (using two pairs
5   of primers), and multiplex amplification (using up to
about 8, 9 or 10 primer pairs).

In preferred embodiments, the PCR process is
used not only to produce a variety of human
Class I-encoding DNA molecules, but also to induce
10   mutations which may emulate those observed in the
highly-polymorphic HLA loci, or to create diversity from
a single parental clone and thereby provide a Class I MHC
molecule-encoding DNA "library" having a greater
heterogeneity. In addition to the mutation inducing
15   variations described in the above referenced U.S. Patent
No. 4,683,195 and such as discussed in U.S. Patent No.
5,314,813.

2.   DNA Expression Vectors

A vector of the present invention is a nucleic
20   acid (preferably DNA) molecule capable of autonomous
replication in a cell and to which a DNA segment, e.g.,
gene or polynucleotide can be operatively linked so as
to bring about replication of the attached segment. One
of the nucleotide segments to be operatively linked to
25   vector sequences is at least a portion of a
mammalian Class I MHC heavy chain. Preferably, the
entire peptide-coding sequence of the MHC heavy chain is
inserted into the vector and expressed; however, it is
also feasible to construct a vector which also includes
30   some non-coding MHC sequences as well. Preferably,
non-coding sequences of MHC are excluded. Alternatively,
a nucleotide sequence for a soluble ("sol") form of an
Class I MHC heavy chain may be utilized; the "sol" form
differs from the non-sol form in that it contains a
35   "stop" codon inserted at the end of the alpha 3 domain or

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prior to the transmembrane domain. Another preferred
vector includes a nucleotide sequence encoding at least a
portion of a mammalian β-2 microglobulin molecule
operatively linked to the vector for expression. Still

5   another preferred vector includes a nucleotide sequence
encoding at least a portion of a mammalian assisting
molecule operably linked to the vector for expression.
It is also feasible to construct a vector including
nucleotide sequences encoding a Class I MHC heavy chain

10   and a β-2 microglobulin and an assisting molecule, or
some combination of these.
        A preferred vector comprises a cassette that
includes one or more translatable DNA sequences
operatively linked for expression via a sequence of

15   nucleotides adapted for directional ligation. The
cassette preferably includes DNA expression control
sequences for expressing the polypeptide or protein that
is produced when a translatable DNA sequence is
directionally inserted into the cassette via the sequence

20   of nucleotides adapted for directional ligation. The
cassette also preferably includes a promoter sequence
upstream from the translatable DNA sequence, and a
polyadenylation sequence downstream from the mammalian
MHC heavy chain sequence. The cassette may also include

25   a selection marker, albeit it is preferred that such a
marker be encoded in a nucleotide sequence operatively
linked to another expression vector sequence.
        The choice of vector to which a cassette of
this invention is operatively linked depends directly, as

30   is well known in the art, on the functional properties
desired, e.g., vector replication and protein expression,
and the host cell to be transformed, these being
limitations inherent in the art of constructing
recombinant DNA molecules.

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        In various embodiments, a vector is utilized
for the production of polypeptides useful in the present
invention, including MHC variants and antigenic peptides.
Exemplary vectors include the plasmids pUC8, pUC9, pUC18,
5   pBR322, and pBR329 available from BioRad Laboratories
(Richmond, CA), pPL and pKK223 available from Pharmacia
(Piscataway, NJ), and pBS and M13mp19 (Stratagene, La
Jolla, CA). Other exemplary vectors include pCMU
(Nilsson, et al., Cell 58: 707 (1989)). Other
10   appropriate vectors may also be synthesized, according to
known methods; for example, vectors pCMU/K" and pCMUII
used in various applications herein are modifications of
pCMUIV (Nilsson, et al., supra).
        In addition, there is preferably a sequence
15   upstream of the translatable nucleotide sequence encoding
a promoter sequence. Preferably, the promoter is
conditional (e.g., inducible). A preferred conditional
promoter used herein is a metallothionein promoter or a
heat shock promoter.
20   Vectors may be constructed utilizing any of the
well-known vector construction techniques. Those
techniques, however, are modified to the extent that the
translatable nucleotide sequence to be inserted into the
genome of the host cell is flanked "upstream" of the
25   sequence by an appropriate promoter and, in some
variations of the present invention, the translatable
nucleotide sequence is flanked "downstream" by a
polyadenylation site. This is particularly preferred
when the "host" cell is an insect cell and the nucleotide
30   sequence is transmitted via transfection. Transfection
may be accomplished via numerous methods, including the
calcium phosphate method, the DEAE-dextran method, the
stable transfer method, electroporation, or via the
liposome mediation method. Numerous texts are available
35   which set forth known transfection methods and other

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procedures for introducing nucleotides into cells; see,
e.g., <u>Current Protocols in Molecular Biology</u>, John Wiley
& Sons, NY (1991).

The vector itself may be of any suitable type,
5   such as a viral vector (RNA or DNA), naked straight-chain
or circular DNA, or a vesicle or envelope containing the
nucleic acid material and any polypeptides that are to be
inserted into the cell. With respect to vesicles,
techniques for construction of lipid vesicles, such as
10   liposomes, are well known. Such liposomes may be
targeted to particular cells using other conventional
techniques, such as providing an antibody or other
specific binding molecule on the exterior of the
liposome. See, e.g., A. Huang, et al., <u>J. Biol. Chem.</u>
15   <u>255</u>: 8015-8018 (1980). See, e.g., Kaufman, <u>Meth.</u>
<u>Enzymol. 185</u>: 487-5:: (1990).

In a preferred embodiment, the vector also
contains a selectable marker. After expression, the
product of the translatable nucleotide sequence may then
20   be purified using antibodies against that sequence. One
example of a selectable marker is neomycin resistance. A
plasmid encoding neomycin resistance, such as phshsneo,
phsneo, or pcopneo may be included in each transfection
such that a population of cells that express the gene(s)
25   of choice may be selected by growing the transfectants
in selection medium

A preferred vector for use according to the
present invention is a plasmid; more preferably, it is a
high-copy-number plasmid. It is also desirable that the
30   vector contain an inducible promoter sequence, as
inducible promoters tend to limit selection pressure
against cells into which such vectors (which are often
constructed to carry non-native or chimeric nucleotide
sequences) have been introduced. It is also preferable

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in the chosen host. If the host cell population is a
Drosophila cell culture, then a compatible vector
includes vectors functionally equivalent to those such as
p25-lacZ (see Bello and Couble, <u>Nature 346</u>: 480 (1990))
5   or pRmHa-1, -2, or -3 (see Bunch, et al., <u>Nucl. Acids
<u>Res. 16</u>: 1043-1061 (1988)). In the preferred embodiment,
the vector is pRmHa-3, which is shown in Figure 3. This
vector includes a metallothionein promoter, which is
preferably upstream of the site at which the MHC sequence
10   is inserted, and the polyadenylation site is preferably
downstream of said MHC sequence. Insect cells and, in
particular, *Drosophila* cells are preferred hosts
according to the present invention. *Drosophila* cells
such as Schneider 2 (S2) cells have the necessary
15   trans-acting factors required for the activation of the
promoter and are thus even more preferred.

The expression vector pRmHa-3 is based on the
bacterial plasmid pRmHa-1 (Figure 2), the latter of which
is based on plasmid pUC18 and is deposited with the
20   American Type Culture Collection (ATCC, Rockville, MD),
having the accession number 37253. The pRmHa-3 vector
contains the promoter, the 5' untranslated leader
sequence of the metallothionein gene (sequences 1-421,
SEQ ID NO 13) with the R1 and Stu sites removed; see
25   Figure 3). It also contains the 3' portion of the
Drosophila ADH gene (sequence #6435-7270, SEQ ID NO 14)
including the polyadenylation site. Therefore, cloned DNA
will be transcriptionally regulated by the
metallothionein promoter and polyadenylated.
30   Construction of the pRmHa-1 plasmid is described in
Bunch, et al., <u>Nucl. Acids Res. 16</u>: 1043-1061 (1988).
Construction of the pRmHa-3 and pRmHa-2 plasmids (the
latter of which has a metallothionein promoter sequence
that may be removed as an Eco RI fragment) is illustrated
35   in Figures 1, 2, and 3. With regard to pRmHa-3, a

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preferred plasmid for use according to the present
invention, Pst I, Sph I and Hind III are in the promoter
fragment and therefore are not unique. Xba is in the ADH
fragment (4 bases from its 3' end) and is also not
5   unique. The following restriction sites are, however,
unique in pRmHa-3: Eco RI, Sac I, Kpn I, Sma I, Bam HI,
Sal I, Hinc 2, and Acc I.

A cassette in a DNA expression vector of this
invention is the region of the vector that forms, upon
10   insertion of a translatable DNA sequence, a sequence of
nucleotides capable of expressing, in an appropriate
host, a fusion protein of this invention. The
expression-competent sequence of nucleotides is referred
to as a cistron. Thus, the cassette preferably comprises
15   DNA expression control elements operatively linked to one
or more translatable DNA sequences. A cistron is formed
when a translatable DNA sequence is directionally
inserted (directionally ligated) between the control
elements via the sequence of nucleotides adapted for that
20   purpose. The resulting translatable DNA sequence, namely
the inserted sequence, is, preferably, operatively linked
in the appropriate reading frame.

DNA expression control sequences comprise a set
of DNA expression signals for expressing a structural
25   gene product and include both 5' and 3' elements, as is
well known, operatively linked to the cistron such that
the cistron is able to express a structural gene product.
The 5' control sequences define a promoter for initiating
transcription and a ribosome binding site operatively
30   linked at the 5' terminus of the upstream translatable
DNA sequence.

Thus, a DNA expression vector of this invention
provides a system for cloning translatable DNA sequences
into the cassette portion of the vector to produce a

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cistron capable of expressing a fusion protein of this
invention.

3.   Cell Lines

A preferred cell line of the present invention
5   is capable of continuous growth in culture and capable of
expressing mammalian Class I MHC molecules and assisting
molecules on the surface of its cells. Any of a variety
of transformed and non-transformed cells or cell lines
are appropriate for this purpose, including bacterial,
10   yeast, insect, and mammalian cell lines. (See, e.g.,
Current Protocols in Molecular Biology, John Wiley &
Sons, NY (1991), for summaries and procedures for
culturing and using a variety of cell lines, e.g., E.
coli and S. cerevisiae.)

15   Preferably, the cell line is a eukaryotic cell
line. More preferably, the cell line is poikilothermic
(i.e., less sensitive to temperature challenge than
mammalian cell lines). More preferably, it is an insect
cell line. Various insect cell lines are available for
20   use according to the present invention, including moth
(ATCC CCL 80), armyworm (ATCC CRL 1711), mosquito larvae
(ATCC lines CCL 125, CCL 126, CRL 1660, CRL 1591, CRL
6585, CRL 6586) and silkworm (ATCC CRL 8851). In a
preferred embodiment, the cell line is a Drosophila cell
25   line such as a Schneider cell line (see Schneider, J.
Embryol. Exp. Morph. 27: 353-365 (1972)); preferably, the
cell line is a Schneider 2 (S2) cell line (S2/M3) adapted
for growth in M3 medium (see Lindquist, et al.,
Drosophila Information Service 58: 163 (1982)).

30   Schneider cells may be prepared substantially
as follows. Drosophila melanogaster (Oregon-R) eggs are
collected over about a 4 hour interval and are
dechlorinated in 2.5% aqueous sodium hypochlorite and
surface-sterilized by immersion in 70% ethanol for 20
35   minutes, followed by an additional 20 minutes in 0.05%

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HgCl₂ in 70% ethanol. After being rinsed thoroughly in
sterile distilled water, the eggs are transferred to
petri dishes containing sterile Metricel black filters
backed with Millipore prefilters, both previously wetted
5   with culture medium. The eggs are placed overnight in a
22 °C incubator and removed for culturing when 20-24
hours old. The embryos are each cut into halves or
thirds, then placed in 0.2% trypsin (1:250, Difco) in
Rinaldini's salt solution (Rinaldini, Nature (London)
10   173: 1134-1135 (1954)) for 20-45 minutes at room
temperature. From 100-300 embryos are used to initiate
each culture.
        After the addition of fetal bovine serum (FBS),
the fragments are centrifuged at 100 X g for 2-3 minutes,
15   resuspended in 1.25ml culture medium and seeded into
glass T-9 flasks. The cultures are maintained at about
22-27 °C ± 0.5 °C, with a gaseous phase of ambient air.
Schneider's culture medium (Schneider, J. Exp. Zool. 156:
91-104 (1964); Schneider, J. Embryol. Exp. Morph. 15:
20   271-279 (1966)) containing an additional 500mg
bacteriological peptone per 100ml medium and supplemented
with 15% inactivated FBS is preferably used. The pH
(preferably 6.7-6.8) is monitored with 0.01% phenol red.
The cell lines are preferably maintained by subculturing
25   every 3-7 days. The cells readily attach to the glass
but not so firmly as to require trypsin treatment;
typically, simple pipetting is adequate to flush most of
the cells from the bottom of the flasks. The
morphological appearance of the cells is described in
30   Schneider, J. Embryol. Exp. Morph. 27: 353-365 (1972).
They are essentially epithelial-like in appearance and
range from about 5-11μm in diameter and 11-35μm in
length. Small pockets containing rounded cells may be
dispersed randomly throughout the other cells.

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Preferably, the Schneider 2 (S2) cells are
maintained in Schneider's *Drosophila* medium plus 10% FBS
including penicillin (100 unit/ml) and streptomycin
(100mg/ml). It is preferable to keep the cells at a
5   density of more than 0.5 X 10⁵/ml, and to grow them at a
24-30 °C temperature range. The cells tend to double in
fewer than 24 hours and grow to high cell density, i.e.,
about 2 X 10⁷/ml or greater. The cells may also be
frozen in 90% FBS and 10% DMSO, for later use or
10   analysis. One may place the cells at -70 °C and then
store in liquid nitrogen.

A preferred cell line according to the present
invention, identified as Schneider 2 (S2) cells, has been
deposited pursuant to Budapest Treaty requirements with
15   the American Type Culture Collection (ATCC), Rockville,
MD, on February 18, 1992, and was assigned accession
number CRL 10974.

Cells of the present invention are transfected
with cDNAs encoding (human) MHC heavy chains, β-2
20   microglobulin and one or more assisting molecules, which
have each been inserted into (i.e., operatively linked
to) an expression vector. In a more preferred
embodiment, the vector comprises *Drosophila* expression
plasmid pRmHa-3, into which expressible nucleotide
25   sequences encoding human Class I MHC heavy chains, human
β-2 microglobulin or human assisting molecules have been
inserted using techniques disclosed herein. Preferably,
the cDNAs encoding MHC heavy chains, those encoding β-2
microglobulin and those encoding assisting molecules are
30   operatively linked to separate expression plasmids and
are cotransfected into the cultured cells.
Alternatively, the cDNAs encoding MHC heavy chains, β-2
microglobulin and assisting molecules may be operatively
linked to the same expression plasmid and cotransfected
35   via that same plasmid. In another variation, cDNAs

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encoding MHC heavy chains, β-2 microglobulin, assisting
molecules, and a cytokine such as IL-2 are operatively
linked to expression plasmids and are cotransfected into
a cell line of the present invention. Selection of HLA
5   genes, construction of appropriate vectors and primer
selection are described in greater detail above.

Successfully transformed cells, i.e., cells
that contain an expressible human nucleotide sequence
according to the present invention, can be identified via
10   well-known techniques. For example, cells resulting from
the introduction of a cDNA or rDNA of the present
invention can be cloned to produce monoclonal colonies.
Cells from those colonies can be harvested, lysed, and
their DNA content examined for the presence of the rDNA
15   using a method such as that described by Southern, J.
Mol. Biol. 98: 503 (1975). In addition to directly
assaying for the presence of rDNA, successful
transformation or transfection may be confirmed by
well-known immunological methods when the rDNA is capable
20   of directing the expression of a subject chimeric
polypeptide. For example, cells successfully transformed
with an expression vector may produce proteins displaying
particular antigenic properties which are easily
determined using the appropriate antibodies. In
25   addition, successful transformation/transfection may be
ascertained via the use of an additional vector bearing a
marker sequence, such as neomycin resistance, as
described hereinabove.

It is also preferable that the culture be
30   stabile and capable of sustained growth at reduced
temperatures. For example, it is preferred that the
culture be maintained at about room temperature, e.g.,
about 24-27 °C. In other embodiments, the culture is
maintained at higher temperatures, particularly during

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preferred that a culture according to the present
invention be capable of withstanding a temperature
challenge of about 30 °C to about 37 °C. Addition of β-2
microglobulin to a culture stabilizes the Class I MHC to

5   at least a 30 °C challenge; addition of β-2 microglobulin
and peptides results in greater thermostability at higher
temperatures, i.e., at 37 °C.

In order to prepare the culture for expression
of empty -- or more preferably, peptide-loaded -- MHC

10   molecules, the culture may first require stimulation,
e.g., via $CuSO_4$ induction, for a predetermined period of
time. After a suitable induction period -- e.g., about
12-48 hours, peptides may be added at a predetermined
concentration (e.g., about 100μg/ml). Peptides may be

15   prepared as discussed below. After a further incubation
period -- e.g., for about 12 hours at 27 °C -- the
culture is ready for use in the activation of CD8⁻ cells.
While this additional incubation period may be shortened
or perhaps omitted, the culture tends to become

20   increasingly stable to temperature challenge if it is
allowed to incubate for a time prior to addition of
resting or naive CD8⁻ cells. For example, cultures
according to the present invention to which peptide has
been added are capable of expressing significant amounts

25   of peptide-loaded Class I MHC molecules even when
incubated for extended periods of time at 37 °C.

Nutrient media useful in the culturing of
transformed host cells are well known in the art and can
be obtained from numerous commercial sources. In

30   embodiments wherein the host cell is mammalian, a
"serum-free" medium is preferably used.

4.   Human β-2 microglobulin and Assisting
Molecules

In order to establish a cell line capable of

35   producing therapeutically useful amounts of

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surface-expressed human Class I MHC molecules, it is
preferable to cotransfect a cell line of the present
invention with a vector operably linked to a nucleotide
sequence encoding β-2 microglobulin in order to effect
5   appropriate levels of expression of human MHC molecules
in the cell line. While the nucleotide sequence encoding
mammalian β-2 microglobulin such as mouse β-2
microglobulin increases the stability of the human
Class I MHC molecules expressed in the cell lines of the
10 present invention, it is preferable to cotransfect the
cell line with a vector operably linked to an expressible
nucleotide sequence encoding a human β-2 microglobulin.

        As discussed above, a preferred vector
according to the present invention includes a nucleotide
15 sequence encoding at least a portion of a mammalian β-2
microglobulin molecule operatively linked to the vector
for expression. The gene for the assisting molecules can
be linked to the same or another vector. It is also
feasible to construct a vector including nucleotide
20 sequences encoding both a Class I MHC heavy chain and a
β-2 microglobulin.

        The sequencing and primers used for the
assisting molecules are discussed in more detail below.
However, the protocols are similar.

25   A human β-2 microglobulin cDNA sequence has
been published (see Suggs, et al., PNAS 78: 6613-17,
1981) and the sequence was used as a template for a
polymerase chain reaction (PCR) using the following
primers:

30 5' primer:

    5' GCTTGGATCCAGATCTACCATGTCTCGCTCCGTGGCCTTAGCTGTGCT
    CGCGCTACTCTC 3'

    (SEQ ID NO 15)

    3' primer

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(SEQ ID NO 16)

The primers are used in a standard PCR reaction (see
above and references cited therein). The reaction
5   products are extracted with phenol, purified using a
Geneclean kit (Bio 101, San Diego, CA), digested with Bam
HI and cloned into the Bam HI site of pBS (Stratagene, La
Jolla, CA). After verification of the sequence, this Bam
HI fragment is cloned into the Bam HI site of an
10   appropriate expression vector. In the preferred
embodiment, human β-2 microglobulin cDNA is synthesized
and operably linked to expression vector pRmHa-3.

5.   Peptides

Virtually all cellular proteins in addition to
15   viral antigens are capable of being used to generate
relevant peptide fragments that serve as potential
Class I MHC ligand. In most mammalian cells, then, any
particular MHC peptide complex would represent only a
small proportion of the total MHC encoded molecules found
20   on the cell surface. Therefore, in order to produce
surface-expressed human Class I MHC molecules that have
an increased capacity to specifically activate CD8$^{\cdot}$
cells, it is preferable to isolate and load peptide
fragments of appropriate size and antigenic
25   characteristics onto Class I molecules.

The peptides of the present invention bind to
Class I MHC molecules. The binding occurs under
biological conditions which can be created in vivo as
well as in vitro. The exact nature of the binding of the
30   peptides need not be known for practice of the invention.

In a preferred embodiment, the peptides to be
loaded onto the Class I MHC molecules are antigenic. It
is also preferred that the peptides be of a uniform size,
preferably 8-mers or 9-mers, and most preferably, 8-mers.
It is also preferable that the peptides prepared for

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loading onto the MHC molecules be of a single species;
i.e., that all peptides loaded onto the MHC be identical
in size and sequence. In this manner, it is possible to
produce monoantigenic peptide-loaded MHC molecules.

5   Peptides may be presented to the cells via
various means. Preferably, peptides are presented in a
manner which allows them to enter an intracellular pool
of peptides. For example, peptides may be presented via
osmotic loading. Typically, peptides are added to the
10   culture medium. The peptides may be added to the culture
in the form of an intact polypeptide or protein which is
subsequently degraded via cellular processes, e.g., via
enzymatic degradation. Alternatively, the intact
polypeptide or protein may be degraded via some other
15   means such as chemical digestion (e.g. cyanogen bromide)
or proteases (e.g. chymotrypsin) prior to its addition to
the cell culture. In other embodiments, the peptides are
presented in smaller segments which may or may not
comprise epitopic amino acid sequences.

20   In a preferred embodiment, a sufficient amount
of protein(s) or peptide(s) is added to the cell culture
to allow the Class I MHC molecules to bind and
subsequently present a large density of the peptide --
preferably, with the same kind of peptide attached to
25   each MHC -- on the surface of human Class I
MHC-expressing cells of the present invention. It is
also preferred to allow the human Class I MHC heavy
chains and human β-2 microglobulin to bind -- i.e., to
form heterodimers -- prior to presenting peptide to the
30   MHC molecules intracellularly.

          In another embodiment of the invention,
peptides are added to transfected cells of the present
invention in order to enhance the thermostability of the
MHC molecules expressed by the cells. As noted above,

Antigenic peptides that bind to the Class I molecules
serve to thermostabilize the MHC molecules and also
increase the cell surface expression. Cultures with
added peptides which bind to the MHC molecules are thus
5   significantly less susceptible to temperature challenge
than cultures without added peptide.

          In one embodiment of the present invention,
antigenic peptides are presented to the
transformed/transfected cell line in various forms. For
10   example, an entire protein or other antigenic polypeptide
may be degraded chemically or enzymatically, for example,
and added to the cell line in this form. For example, a
protein of interest is degraded with chymotrypsin and the
resultant mixture of peptide "fragments" is added to a
15   transformed or transfected cell culture; these cells are
then allowed to "choose" the appropriate peptides (which
are often smaller peptides, preferably 8mers or 9mers) to
load onto the Class I MHC molecules. Alternatively, an
entire protein or polypeptide sequence may be cloned into
20   an appropriate vector and inserted into a procaryotic
cell, whereby the cell generates significant amounts of
the antigenic polypeptide which are then harvested,
purified, and digested into peptides which are then added
to the transformed/transfected eukaryotic cell culture.
25   The cells again would be allowed to "choose" the peptides
to load onto the expressed MHC.

          6.   Isolation of Resting or Precursor CD8·
cells

          Resting (or naive or precursor) CD8· cells --
30   i.e., T-cells that have not been activated to target a
specific antigen -- are preferably extracted from the
patient prior to incubation of the CD8· cells with the
transformed cultures of the present invention. It is
also preferred that precursor CD8· cells be harvested
35   from a patient prior to the initiation of other treatment

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or therapy which may interfere with the CD8 cells'
ability to be specifically activated. For example, if
one is intending to treat an individual with a neoplasia
or tumor, it is preferable to obtain a sample of cells
5   and culture same prior to the initiation of chemotherapy
or radiation treatment.

       Methods of extracting and culturing lymphocytes
are well known. For example, U.S. Patent No. 4,690,915
to Rosenberg describes a method of obtaining large
10   numbers of lymphocytes via lymphocytopheresis.
Appropriate culturing conditions used are for mammalian
cells, which are typically carried out at 37 °C.

       Various methods are also available for
separating out and/or enriching cultures of precursor
15   CD8 cells. Some examples of general methods for cell
separation include indirect binding of cells to
specifically-coated surfaces. In another example, human
peripheral blood lymphocytes (PBL), which include CD8
cells, are isolated by Ficoll-Hypaque gradient
20   centrifugation (Pharmacia, Piscataway, NJ). PBL
lymphoblasts may be used immediately thereafter or may be
stored in liquid nitrogen after freezing in FBS
containing 10% DMSO (Sigma Chemical Co., St. Louis, MO),
which conserves viability and lymphocyte functions.
25   Alternative methods of separating out and/or
enriching cultures of precursor cells include both
positive and negative selection procedures. For positive
selection, after lymphocyte-enriched PBL populations are
prepared from whole blood, sub-populations of CD8
30   lymphocytes are isolated therefrom by affinity-based
separation techniques directed at the presence of the CD8
receptor antigen. These affinity-based techniques
include flow microfluorimetry, including
fluorescence-activated cell sorting (FACS), cell

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in <u>Fundamental Immunology</u>, W.E. Paul, ed., pp. 767-780,
River Press, NY (1984).) Affinity methods may utilize
anti-CD8 receptor antibodies as the source of affinity
reagent. Alternatively, the natural ligand, or ligand
5   analogs, of CD8 receptor may be used as the affinity
reagent. Various anti-T-cell and anti-CD8 monoclonal
antibodies for use in these methods are generally
available from a variety of commercial sources, including
the American Type Culture Collection (Rockville, MD) and
10   Pharmingen (San Diego, CA).

Negative selection procedures are utilized to
effect the removal of non-CD8 from the CD8⁺ population.
This technique results in the enrichment of CD8⁺ cells
from the T- and B-cell population of leucophoresed
15   patients. Depending upon the antigen designation,
different antibodies may be appropriate. (For a
discussion and review of nomenclature, antigen
designation, and assigned antibodies for human
leucocytes, including T-cells, see Knapp, et al.,
20   <u>Immunology Today 10</u>: 253-258 (1989) and Janeway et al.,
<u>Immunobiology, supra</u>.) For example, monoclonal
antibodies OKT4 (anti-CD4, ATCC No. CRL 8002) OKT 5 (ATCC
Nos. CRL 8013 and 8016), OKT 8 (anti-CD8, ATCC No. CRL
8014), and OKT 9 (ATCC No. CRL 8021) are identified in
25   the ATCC Catalogue of Cell Lines and Hybridomas (ATCC,
Rockville, MD) as being reactive with human T
lymphocytes, human T-cell subsets, and activated T-cells,
respectively. Various other antibodies are available for
identifying and isolating T-cell species.
30   In a further embodiment, CD8⁺ cells can be
isolated by combining both negative and positive
selection procedures. (See, e.g. Cai and Sprent, <u>J. Exp.</u>
<u>Med. 179</u>: 2005-2015 (1994)).

Preferably, the PBLs are then purified. For
35   example, Ficoll gradients may be utilized for this

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purpose. The purified PBLs would then be mixed with
syngeneic *Drosophila* cells preincubated with the
appropriate antigenic peptides.

       7.   *In Vitro Activation of CD8· Cells*

5   In order to optimize the *in vitro* conditions
for the generation of specific cytotoxic T-cells, the
culture of antigen-presenting cells is maintained in an
appropriate medium. In the preferred embodiment, the
antigen-presenting cells are *Drosophila* cells, which are
10 preferably maintained in serum-free medium (e.g. Excell
400).

       Prior to incubation of the antigen-presenting
cells with the cells to be activated, e.g., precursor
CD8· cells, an amount of antigenic peptide is added to
15 the antigen-presenting cell culture, of sufficient
quantity to become loaded onto the human Class I
molecules to be expressed on the surface of the
antigen-presenting cells. According to the present
invention, a sufficient amount of peptide is an amount
20 that will allow about 200 to about 500,000 and preferably
about 200 to 1,000 or more, human Class I MHC molecules
loaded with peptide to be expressed on the surface of
each antigen-presenting cell. Preferably, the
antigen-presenting cells are incubated with >20µg/ml
25 peptide.

       Resting or precursor CD8· cells are then
incubated in culture with the appropriate
antigen-presenting cells for a time period sufficient to
activate and further enrich for a population of CD8·
30 cells. Preferably, the CD8· cells shall thus be
activated in an antigen-specific manner. The ratio of
resting or precursor CD8· (effector) cells to
antigen-presenting cells may vary from individual to
individual and may further depend upon variables such as

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culturing conditions and the nature and severity of the
disease condition or other condition for which the
within-described treatment modality is used. Preferably,
however, the lymphocyte:antigen-presenting cell (e.g.
5   Drosophila cell) ratio is preferably in the range of
about 30:1 to 300:1. For example, in one embodiment, 3 X
10^7 human PBL and 1 X 10^6 live Drosophila cells were
admixed and maintained in 20 ml of RPMI 1640 culture
medium.

10   The effector/antigen-presenting culture may be
maintained for as long a time as is necessary to activate
and enrich for a population of a therapeutically useable
or effective number of CD8· cells. In general terms, the
optimum time is between about one and five days, with a
15   "plateau" -- i.e. a "maximum" specific CD8· activation
level -- generally being observed after five days of
culture. In one embodiment of the present invention, in
vitro activation of CD8· cells is detected within a brief
period of time after transfection of a cell line. In one
20   embodiment, transient expression in a transfected cell
line capable of activating CD8· cells is detectable
within 48 hours of transfection. This clearly indicates
that either stable or transient cultures of transformed
cells expressing human Class I MHC molecules are
25   effective in activating CD8· cells.
                Preferably, the enrichment and concordant
activation of CD8· cells is optimal within one week of
exposure to antigen-presenting cells. Thereafter, in a
preferred embodiment, the enriched and activated CD8·
30   cells are further purified by isolation procedures
including site restriction, rosetting with antibody-red
blood cell preparations, column chromatography and the
like. Following the purification, the resulting CD8·
cell preparation is further expanded by maintenance in
35   culture for a period of time to obtain a population of

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10$^?$ activated CD8$^.$ cells. This period may vary depending
on the replication time of the cells but may generally be
14 days. Activation and expansion of CD8$^.$ cells has been
described by Riddell et al., <u>Curr. Opin. Immunol.</u>, 5:
5   484-491 (1993).

       8.   <u>Separation of CD8$^.$ Cells</u>
           <u>from Drosophila Cells</u>

       Activated CD8$^.$ cells may be effectively
separated from the stimulator (e.g., Drosophila) cells
10   using one of a variety of known methods. For example,
monoclonal antibodies specific for the stimulator cells,
for the peptides loaded onto the stimulator cells, or for
the CD8$^.$ cells (or a segment thereof) may be utilized to
bind their appropriate complementary ligand.
15   Antibody-tagged cells may then be extracted from the
stimulator-effector cell admixture via appropriate means,
e.g., via well-known immunoprecipitation or immunoassay
methods.

       9.   <u>Administration of Activated CD8$^.$ Cells</u>

20   Effective, cytotoxic amounts of the activated
CD8$^.$ cells can vary between <i>in vitro</i> and <i>in vivo</i> uses, as
well as with the amount and type of cells that are the
ultimate target of these killer cells. The amount will
also vary depending on the condition of the patient and
25   should be determined via consideration of all appropriate
factors by the practitioner. Preferably, however, about
1 X 10$^.$ to about 1 X 10^{12}, more preferably about 1 X 10$^+$
to about 1 X 10^{11}, and even more preferably, about 1 X 10$^.$
to about 1 X 10^{10} activated CD8$^.$ cells are utilized for
30   adult humans, compared to about 5 X 10$^.$ - 5 X 10$^.$ cells
used in mice.

       Preferably, as discussed above, the activated
CD8$^.$ cells are harvested from the Drosophila cell culture
prior to administration of the CD8$^.$ cells to the

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however, that unlike other present and proposed treatment
modalities, the present method uses a cell culture system
(i.e., *Drosophila* cells) that are not tumorigenic.
Therefore, if·complete separation of *Drosophila* cells and
5   activated CD8· cells is not achieved, there is no
inherent danger known to be associated with the
administration of a small number of *Drosophila* cells,
whereas administration of mammalian tumor-promoting cells
may be extremely hazardous.

10   Methods of re-introducing cellular components
are known in the art and include procedures such as those
exemplified in U.S. Patent No. 4,844,893 to Honsik, et
al. and U.S. Patent No. 4,690,915 to Rosenberg. For
example, administration of activated CD8· cells via
15   intravenous infusion is appropriate.

          10. HLA Typing

          As noted previously, HLA haplotypes/allotypes
vary from individual to individual and, while it is not
essential to the practice of the present invention, it is
20   often helpful to determine the individual's HLA type.
The HLA type may be determined via standard typing
procedures and the PBLs purified by Ficoll gradients.
The purified PBLs would then be mixed with syngeneic
Drosophila cells preincubated with the appropriate
25   antigenic peptides -- e.g., in therapeutic applications
relating to viral infections, cancers, or malignancies,
peptides derived from viral- or cancer-specific proteins.

          Continuing to use viral or malignant conditions
as an example, in those instances in which specific
30   peptides of a particular viral- or cancer-specific
antigen have been characterized, the synthesized peptides
encoding these epitopes will preferably be used. In
cases in which the preferred antigenic peptides have not
been precisely determined, protease digests of viral- or
35   cancer-specific proteins may be used. As a source for

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such antigen, cDNA encoding viral- or cancer-specific
proteins is cloned into a bacterial expression plasmid
and used to transform bacteria, e.g., via methods
disclosed herein.

5   After HLA typing, if *Drosophila* cells
expressing the preferred HLA are not available, cDNAs
encoding the preferred HLA may be cloned via use of the
polymerase chain reaction. The primers disclosed in
section B.1. above (SEQ ID NO 1 through SEQ ID NO 12) may

10 be used to amplify the appropriate HLA-A, -B, -C, -E, -F,
or -G cDNAs in separate reactions which may then be
cloned and sequenced as described in the methods
disclosed for HLA A2.1 below. Stable cell lines
expressing the cloned HLA may then be established in the

15 *Drosophila* cells. Alternatively, a population of insect
cells transiently expressing a bulk population of cloned
recombinant molecules from the PCR reaction may be used
for *in vitro* CD8⁻ activation.

EXAMPLES

20   The following examples are intended to
illustrate, but not limit, the present invention.

Example 1

Expression of Human Class I MHC Molecules

A.   Preparation of pRmHa-3 Expression Vector

25   The pRmHa-3 expression vector for use in
expressing MHC proteins in *Drosophila* Schneider 2 (S2)
cells as described in this invention was constructed by
ligating a Sph I linearized pRmHa-1 DNA expression vector
with a DNA fragment resulting from a Sph I restriction

30 digest of a pRmHa-2 expression vector as described below.
The ligating of pRmHa-1 with the pRmHa-2 fragment in this
manner was performed to remove one of two Eco RI
restriction endonuclease cloning sites present in
pRmHa-1. Thus, the resultant pRmHa-3 expression vector

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multiple cloning site (polylinker) into which various
MHC-encoding DNA fragments were inserted as described in
the Examples.

1. Preparation of pRmHa-1 Expression Vector

5   The pRmHa-1 expression vector, containing a
metallothionein promoter, metal response consensus
sequences (designated MT) and an alcohol dehydrogenase
(ADH) gene containing a polyadenylation signal isolated
from Drosophila melanogaster, was constructed as

10   described by Bunch et al., Nucl. Acids Res. 16: 1043-61
(1988). A schematic of the final pRmHa-1 construct is
shown in Figure 2. The plasmid expression vector, pUC18,
having the ATCC accession number 37253, was used as the
source vector from which subsequent vectors described

15   herein were derived. The pUC18 plasmid contains the
following restriction sites from 5' to 3' in the multiple
cloning site, all of which are not illustrated in the
schematic representations of the pUC18-derived vectors in
Figure 1: Eco RI; Sac I; Kpn I; Sma I and Sma I located

20   at the same position; Bam HI; Xba I; Sal I, Acc I and
Hinc II located at the same position; Pst I; Sph I and
Hind III. The pUC18 vector was first digested with Hind
III to form a linearized pUC18. Blunt ends were then
created by filling in the Hind III ends with DNA

25   polymerase I large fragment as described by Maniatis et
al., Molecular Cloning: A Laboratory Manual, eds. Cold
Spring Harbor Laboratory, New York (1982).

         The resultant linearized blunt-ended pUC18
vector was ligated with a 740 base pair (bp) Hinf I

30   fragment from the Drosophila melanogaster ADH gene
containing a polyadenylation signal. The ligated ADH
allele was first isolated from the plasmid pSACI,
described by Goldberg et al., PNAS USA 77: 5794-5798
(1980), by digestion with Hinf I followed by blunt ending

35   with Klenow resulting in the nucleotide sequence listed

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in SEQ ID NO 14.   The pSACI vector containing the ADH
allele was constructed by subcloning into pBR322 (ATCC
accession number 31344) a 4.7 kilobase (kb) Eco : :
fragment of *Drosophila* DNA selected from a bacteriophage
5   lambda library containing random, high molecular weight
(greater than 15 kb). The 5' Hinf I restriction site
occurred naturally in the ADH gene at position 1770 as
described by Kreitman, Nature 304: 412-417 (1983). The
3' Hinf I site was derived from the pUC18 vector into
10   which the ADH gene had been cloned. This position was
four bases 3' to the Xba I site at position 2500 of the
ADH gene. The ADH segment extended from the 35 bp
upstream of the polyadenylation/cleavage sequence in the
3' untranslated portion of the ADH mRNA to 700 bp
15   downstream of the polyadenylation signal. The resultant
pUC18-derived vector containing the ADH gene fragment was
designated pHA-1 as shown in Figure 1.
The 421 bp Eco RI/Stu I MT gene fragment was
obtained from a clone containing DNA of approximately
20   15.3 kb in a *Drosophila melanogaster* genomic DNA library.
The library, prepared with a Mbo I partial digestion of
imaginal DNA, was cloned in the lambda derivative EMBL4.
The fragment contained the MT promoter and metal response
consensus elements of the *Drosophila* MT gene (Maroni et
25   al., Genetics 112: 493-504 (1986)). This region,
containing the promoter and transcription start site at
nucleotide 1+, corresponded to position -370 to
nucleotide position +54 of the MT gene (SEQ ID NO 13).
The resultant fragment was then ligated into pHA-1
30   expression vector prepared above that was previously
linearized with Eco RI and Sma I. The 3' blunt end in MT
created by the Stu I digest was compatible with the blunt
end in pHA-1 created by the Sma I digest. The resultant
pUC18-derived vector containing a 5' *Drosophila* MT gene

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pRmHa-1. The pRmHa-1 expression vector, shown in
Figure 2, contained the origin of replication (ori) and
the beta-lactamase gene conferring resistance to
ampicillin (Amp') from pUC18 as shown in Figure 1 on the
5   pHa-1 vector. The diagram of pRmHa-1 also shows the 5'
to 3' contiguous positions of the MT gene fragment, the
multiple cloning site and the ADH gene fragment. The
pRmHa-1 vector was used as described in c. below in the
construction of the pRmHa-3 expression vector.
10   2.   Preparation of pRmHa-2 Expression Vector
            The construction of pRmHa-2 is shown in
Figure 1. For constructing the pRmHa-2 expression
vector, the MT fragment prepared above was inserted into
the pUC18-derived vector pHA-1 as described for
15   constructing pRmHa-1 above with a few modifications.
An Eco RI linker was added to the Stu I site of the Eco
RI/Stu I-isolated MT gene fragment prepared above to form
a metallothionein fragment having Eco RI restriction
sites on both ends. The resultant fragment was then
20   ligated into the ADH fragment-containing pUC18 expression
vector that was previously linearized with Eco RI. The
resultant pUC18-derived vector containing a 5' Drosophila
MT gene fragment and a 3' ADH gene fragment having two
Eco RI restriction sites 5' to the multiple cloning site
25   was designated pRmHa-2. The pRmHa-2 expression vector,
shown in Figure 1, contained the origin of replication
(ori) and the beta-lactamase gene conferring resistance
to ampicillin (Amp') from pUC18. The diagram of pRmHa-2
also shows the 5' to 3' contiguous positions of the MT
30   gene fragment, the multiple cloning site and the ADH gene
fragment. The pRmHa-2 vector was used along with pRmHa-1
as described in c. below in the construction of the
pRmHa-3 expression vector.

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2.   <u>Preparation of pRmHa-3 Expression Vector</u>

        To prepare the pRmHa-3 expression vector that
had only one Eco RI restriction site, a fragment from
pRmHa-2 was ligated into pRmHa-1. For this construction,
5   pRmHa-2, prepared in b. above, was first digested with
Sph I. The resultant Sph I fragment beginning in the
middle of the MT gene and extending to the Sph I site in
the multiple cloning site was first isolated from the
pRmHa-2 vector and then ligated into pRmHa-1 prepared in
10   A.1. above. The pRmHa-1 vector was previously modified
to remove the Eco RI restriction site 5' to the MT gene
fragment then linearized with Sph I. This process is
schematically illustrated in Figure 2. To remove the Eco
RI site in pRmHa-1, the vector was first digested with
15   Eco RI to form a linearized vector, then blunt ended with
Mung Bean nuclease and religated.

        The pRmHa-1 vector lacking an Eco RI site was
then digested with Sph I to remove the region
corresponding to the Sph I fragment insert from pRmHa-2
20   and form a linearized pRmHa-1 vector. The Sph I fragment
from pRmHa-2 was then ligated into the Sph I linearized
pRmHa-1 to form the pRmHa-3 expression vector. A
schematic of the pRmHa-3 vector is shown in Figure 3.
The relative positions of the various restriction sites
25   from the pUC18 vector from which pRmHa-3 was derived are
indicated on the figure. In addition, the relative
positions and lengths of the MT and ADH gene fragments
separated by the multiple cloning site (polylinker) into
which the MHC gene of interest is cloned are indicated on
30   the figure. The pRmHa-3 vector, being derived from
pUC18, contains the pUC18 origin of replication and
beta-lactamase gene conferring ampicillin resistance.
Thus, MHC encoding DNA fragments as prepared in this
invention and cloned into the multiple cloning site of

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pRmHa-3 were transcriptionally regulated by the MT
promoter and polyadenylated via the ADH gene.

    B.   <u>cDNA Synthesis</u>

    Detailed descriptions of the cDNA of Class I
5 MHC molecules of various HLA groups can be found in U.S.
Patent No. 5,314,813 to Peterson et al. which has been
incorporated by reference.

    cDNAs encoding any preferred HLA may be cloned
via use of the polymerase chain reaction. The primers
10 disclosed in section B.1. above (SEQ ID NO 1 through SEQ
ID NO 12) may be used to amplify the appropriate HLA-A,
-B, -C, -E, -F, or -G cDNAs in separate reactions which
may then be cloned and sequenced as described in the
methods disclosed for HLA A2.1 above. Preparation of
15 cDNA from human cells is carried out as described in
Ennis, et al., <u>PNAS USA 87</u>: 2833-2837 (1990). Briefly, a
blood sample is obtained from the individual and cells
are collected after centrifugation and used to prepare
total RNA. First strand cDNA is synthesized by using
20 oligo(dT) and avian myeloblastosis virus reverse
transcriptase. The resulting cDNA is used in a PCR
amplification reaction utilizing the appropriate
primer(s) as noted in section B.1. above, and a GeneAmp
kit and thermal cycler (Perkin-Elmer/Cetus). Reaction
25 conditions are typically as follows. 100ng cDNA
template and 5 picomoles of each oligonucleotide primer
are used. Thirty cycles are run as follows: (a) one
minute at 94 °C; (b) one minute at 60 °C; and (c) one
minute, 30 seconds at 72 °C. The PCR reaction is then
30 heated to 100 °C for 10 minutes to kill the Taq
polymerase and the ends of the DNA made blunt by T4
polymerase (Stratagene, San Diego, CA).

    To synthesize HLA A2.2, cDNA encoding a
complete A2.2 (see Holmes, et al., <u>J. Immunol. 139</u>:
35 936-41 (1987), for the published sequence) is cloned into

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an M13mp19 plasmid, a commercially available
bacteriophage vector (Stratagene, La Jolla, CA). cDNA is
synthesized by PCR using primers derived from the
published sequence of A2. The cDNA is released from an
5   M13mp19 clone as a Not I (overhang filled with
Klenow)/Eco RI fragment. (Klenow fragments are part of
the *E. coli* DNA polymerase I molecule, produced by the
treatment of *E. coli* DNA pol I with subtilisin. They are
used to "fill out" 5' or 3' overhangs at the ends of DNA
10   molecules produced by restriction nucleases.) The Not
I/Eco RI fragment is inserted into pSP64T digested with
Bg III (ends filled with Klenow) and Eco RI. pSP64T is
an SP6 cloning vector designed to provide 5' and 3'
flanking regions from an mRNA which is efficiently
15   translated (β-globin) to any cDNA which contains its own
initiation codon. This translation SP6 vector was
constructed by digesting pSP64-Xβm with Bal I and Bst
EII, filling in the staggered ends with T4 DNA polymerase
and adding a Bgl II linker by ligation. Bal I cuts the
20   β-globin cDNA two bases upstream of the ATG (start codon)
and Bst EII cuts eight bases upstream of the TAA (stop
codon). There is only one Bgl II site in pSP64T so that
restriction enzymes cutting in the polylinker fragment,
from Pst I to Eco RI can still be used to linearize the
25   plasmid for transcription. (See Kreig and Melton,
<u>Nucleic Acid Res. 12</u>: 7057-7070, (1984), which also
describes the construction of the plasmid pSP64-Xβm.)
The resulting plasmid is cleaved with Eco RI (end filled
with Klenow) and Hind III which is cloned into the pCMUII
30   polylinker between Hind III (5') and Stu I (3'). (See
Paabo, et al., <u>EMBO J. 5</u>: 1921-1927 (1986).) The entire
cDNA is removed as a Hind III (end filled with Klenow)
Bam HI fragment which is cloned into pRmHa-3 cleaved with
Sma I and Bam HI.

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        HLA A2.2 soluble form was prepared by
engineering a stop codon into the above-described A2.2
cDNA immediately preceding the transmembrane domain. The
modification is achieved by cleaving the A2.2 cDNA cloned
5   in the eukaryotic expression vector pCMUII between Hind
III 5' and Stu I 3' (see above) with Mbo II and Bam HI
inserting the following oligonucleotides:
        5' primer: 5' GGAGCCGTGACTGACTGAG 3'
(SEQ ID NO 17)
10   **3' primer**: 5' CCCTCGGCACTGACTGACTCCTAG 3'
(SEQ ID NO 18)

The resulting recombinant plasmid is cleaved with Hind
III, the overhanging end filled with Klenow, then cut
15 with Bam HI releasing a restriction fragment which is
cloned into pRmHa-3 in the same way as A2.2 full length.
1. <u>Construction of Murine ICAM-1 Expression Vector</u>
            Spleen cells were isolated from Balb/c mice.
The spleen cells were stimulated with conA; mRNA was
20 isolated using the FastTrack kit (Invitrogen, San Diego,
CA) according to the manufacturers' instructions. cDNA
was synthesized from the mRNA using AMV reverse
transcriptase kit (Promega, Madison, WI) according to the
manufacturers' instructions. Based on the published cDNA
25 nucleotide sequence (Siu, G. et al., J. lmmunol. 143,
3813-3820 (1989) the following oligonucleotides were
synthesized as PCR primers:
5': TTTAGAATTCAC CATGGCTTCA ACCCGTGCCA AG
3': TTTAGTCGACTC AGGGAGGTGG GGCTTGTCC
30 The cDNA synthesized was subjected to PCR using these
primers. The product was cleaved with the restriction
enzymes Eco RI and Sal I and ligated into pRmHa-3, which
had been digested with the restriction enzymes Eco RI and
Sal I.

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2. <u>Construction of Murine B7.1 Expression Vector</u>

Spleen calls were isolated from Balb/c mice and stimulated with conA. Messenger RNA was isolated using the FastTrack kit (Invitrogen, San Diego, CA) according
5 to the manufacturer's instructions. cDNA was synthesized from the mRNA using AMV reverse transcriptase kit (Promega, Madison, WI) according to the manufacturer's instructions.

Based on the published cDNA nucleotide sequence
10 (Freeman, et al., J. Exp. Med. 174: 625-631 (1991)) the following oligonucleotides were synthesized as PCR primers:

5': TTTAGAATTCAC CATGGCTTGC AATTGTCAGT TG
3': TTTAGTCGACCT AAAGGAAGAC GGTCTGTTC

15 The cDNA synthesized was subjected to PCR using these primers. The product was cleaved with the restriction enzymes Eco RI and Sal I and ligated into pRmHa-3, which had been digested with the restriction enzymes Eco RI and Sal I.

20 3. <u>Construction of Murine B7.2 Expression Vector</u>

IC-21 cells obtained from ATCC) were propagated in RPMI 1640 medium containing 10% Fetal Calf Serum. mRNA was isolated from these cells using the FastTrack kit (Invitrogen, San Diego, CA) according to
25 the manufacturer's instructions. cDNA was synthesized from the mRNA using AMV reverse transcriptase kit (Promega, Madison, WI) according to the manufacturer's instructions. Based on the published cDNA nucleotide sequence (Freeman, et al., J. Exp. Med. 178: 2185-2192
30 (1993)) the following oligonucleotides were synthesized as PCR primers:

5': TTTAGAATTCAC CATGGACCCC AGATGCACCA TGGG
3': TTTAGTCGACTC ACTCTGCATT TGGTTTTGCT GA

The cDNA synthesized was subjected to PCR using these

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enzymes Eco RI and Sal I and ligated into pRmHa-3, which had been digested with the restriction enzymes Eco RI and Sal I.

The above expression constructs were transfected into *Drosophila* S2 cells using the calcium phosphate method as listed in Table 1. Stable cell lines were selected by including 500 μg/ml Geneticin in the cell culture medium.

TABLE 1

Transfected Cells		MHC I (L^q) μg	β2 μg	B7.1 (CD 80) μg	B7.2 μg	ICAM-1 (CD54) μg	phsneo μg
1	A	12	12				1
2	B	8	8	8			1
3	C	8	8		8		1
4	C	8	8			8	1
5	D	6	6	6		6	1
6	E	6	6		6	6	1
7	F	6	6	6	6		1
8	G	4.8	4.8	4.8	4.8	4.8	1

Human accessory and costimulatory molecules were cloned from human cell lines demonstrated to express these proteins by FACS analysis with monoclonal antibodies specific for the particular proteins. Adhesion molecules belonging to the integrin family, ICAM-I (CD54) and LFA-3 (CD58), were cloned from human cell lines K562 and HL60, respectively. The K562 cells, originated from human chronic myelogenous leukemia, were obtained from ATCC (CCL-243) and cultured under conditions recommended (i.e., RPMI with 10% fetal calf serum at 37 degrees C with 5% CO$_2$). HL60 cells, originated from a human promyelocytic leukemia, and were obtained from ATCC (CCL-240) and were cultured according

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to ATCC's recommendations. Costimulatory molecules B7.1
and B7.2 were also cloned from K562 and HL60 cells
respectively.

4. cDNA

5   Messenger RNA samples were prepared from each
cell line from RNA isolated by the modified guanidinium
thiocyanate method (Chromczynski, et al. Anal. Biochem.
162: 156-159, 1987) followed by poly A+ RNA selection on
oligo(dt)-cellulose columns (Sambrook, J., et al,

10 Molecular Cloning: A Laboratory Manual, Second Edition,
6.22-6.34, Cold Spring Harbor laboratory, CSH, NY),
Induction of HL60 cells with vitamin D3 (usually required
to express some cell surface molecules) was not required
to obtain the B7.2 and LFA-3 molecules, the proteins were

15 expressed in the absence of induction. cDNA was prepared
using AMV reverse transcriptase kit according to the
manufacturers' instructions (Promega, Madison WI).

5. PCR Primers

          PCR primers were designed and synthesized after
20 obtaining copies of the known sequences from the
GENEWORKS database (Intelligenetics) and considering the
ends needed to clone into the appropriate vectors. They
are as follows with the top sequence of each protein the
5'primer and the bottom one the 3'primer:

25 B7.1   5'-ACCCTTGAAT CCATGGGCCA CACACGGAGG CAG-3'
        5'-ATTACCGGAT CCTTATACAG GGCGTACACT TTCCCTTCT-3'
    B7.2   5'-ACCCTTGAGC TCATGGATCC CCAGTGCACT ATG-3'
        5'-ATTACCCCCG GGTTAAAAAC ATGTATCACT TTTGTCGCAT GA-3'
    LFA-3   5'-ACCCTTGAGC TCATGGTTGC TGGGAGCGAC GCGGGG-3'
30   3'-ATTACCGGAT CCTTAAAGAA CATTCATATA CAGCACAATA CA-3'
    ICAM-1 5'-ACCCTTGAAT TCATGGCTCC CAGCAGCCCC CGGCCC-3'
        3'-ATTACCGGAT CCTCAGGGAG GCGTGGCTTG TGTGTTCGG-3'

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6. Expression of DNA Fragment

         The cDNA preparations from each of the cell
lines was used to clone the desired proteins. The
polymerase chain reaction was used to generate cDNA
5   fragments utilizing the appropriate PCR primer (see
above). The appropriate DNA fragments were cloned into
the Drosophila fly vector pRMHA-3. Plasmid preparations
have been prepared from all of the preparations and are
now ready for transfection into the fly cells.
10   Human β-2 microglobulin cDNA is prepared using
a published partial cDNA sequence (see Suggs, et al.,
PNAS 78: 6613-17, 1981) is used as a template for a
polymerase chain reaction (PCR) with the following
primers:
15   **5' primer**
     5' GCTTGGATCCAGATCTACCATGTCTCGCTCCGTGGCCTTAGCTGTGCTCGC
     GCTACTCTC 3'
     (SEQ ID NO 15)
     3' primer
20   5' GGATCCGGATGGTTACATGTCGCGATCCCACTTAAC 3'
     (SEQ ID NO 16)

     The primers are used in a standard PCR reaction (see
     Nilsson, et al., Cell 58: 707 (1989)). The reaction
25   products are extracted with phenol, purified using a
     Geneclean kit (Bio 101, San Diego, CA), digested with Bam
     HI and cloned into the Bam HI site of pBS (Stratagene, La
     Jolla, CA). After verification of the sequence, this Bam
     HI fragment is cloned into the Bam HI site of pRmHa-3.
30   As noted in the Examples, murine Class I cDNA
     was utilized in various instances. Murine Class I cDNA
     was prepared as follows.
         H-2K": cDNA encoding a complete K" molecule is
     obtained from an expression plasmid pCMU/K" constructed
35   as follows. A partial H-2K" cDNA missing the leader

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sequence and most of the alpha I domain is prepared
according to the method of Reyes, et al., PNAS 79:
3270-74 (1982), producing pH202. This cDNA is used to
generate a full-length molecule. The missing sequence is
5   provided using a genomic clone encoding H-2K^h (Caligan,
et al., Nature 291: 35-39, 1981) as a template in a PCR
reaction, using a 5' primer flanked by a Not I site,
followed by 21 nucleotides encoding the last seven amino
acids of the leader sequence and 18 nucleotides
10   complementary to the beginning of the alpha I domain and
a 3' primer complementary to the region encompassing the
Sty I site. The resulting fragment is ligated with pH202
at the Sty I site. The 5' sequence encoding the
remainder of the signal sequence is obtained form the D^h
15   cDNA (see below) as a Bam HI/Not I fragment. The entire
coding sequence is cleaved from the expression plasmid as
a Bam HI fragment and cloned into pRmHa-3 cleaved with
Bam HI.

H-2L^d: cDNA encoding a complete L^d molecule is
20   obtained from an expression plasmid pCMUIV/L^d (see Joly
and Oldstone, Gene 97: 213, 1991). The complete cDNA is
cleaved from a eukaryotic expression vector pCMU IV/L^d as
a Bam HI fragment and cloned into pRmHa-3 as K^h.

As noted previously, the pCMU vector (pCMUIV)
25   is derived from eukaryotic expression vector pC81G as
described in Nilsson, et al., supra. Vector pC81G, in
turn, is derived from pA81G (Paabo, et al., Cell 33:
445-453 (1983)) according to the method disclosed in
Paabo, et al., EMBO J. 5: 1921-7 (1986).

30   H-2D^b: cDNA encoding a complete D^b molecule is
obtained from expression plasmid pCMUIV/D^b (see Joly and
Oldstone, Science 253: 1283-85, 1991). The complete cDNA
is cleaved from a eukaryotic expression vector pCMUIV/D^b
as a Bam HI fragment and cloned into pRmHa-3 as K^b.

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Murine β-2 microglobulin: full-length murine
β-2 microglobulin cDNA is obtained as a Hind III (5')
(filled with Klenow)/Bgl II (3') fragment from pSV2neo
(ATCC No. 37-149) mouse β-2 microglobulin cDNA and cloned
5   into pRmHa-3 cleaved with Sma I and Bam HI.
Vector phshsneo confers neomycin (G418)
resistance and is a derivative of phsneo (pUChsneo) with
an additional heat-shock promoter (hs) sequence, which
may be synthesized from commercially-available pUC8 as
10   described in Steller, et al., EMBO J. 4: 167 (1985). The
heat shock promoter contained in these vectors is the
hsp70 promoter. Other useful vectors conferring neomycin
resistance (G418 resistance) include cosmid vector smart2
(ATCC 37588), which is expressed under the control of
15   Drosophila hsp70 promoter, and plasmid vector pcopneo
(ATCC 37409).

      C.   Insertion of Genes into Expression Vectors

The restriction products are subjected to
electrophoresis on a 1% agarose gel (Maniatis, et al.,
20   Molecular Cloning: A Laboratory Manual, Cold Spring
Harbor Laboratory (1982)). The restriction fragments
encoding the cDNAs are excised from the gel and purified
away from the agarose using "Geneclean", according to
manufacturers' directions (Bio 101, San Diego, CA). The
25   expression plasmid pRmHa-3 (Figure 3) is cleaved with the
appropriate restriction enzymes in One Phor All buffer
according to the manufacturer's directions (Pharmacia,
Piscataway, NJ) and treated with alkaline phosphatase as
described in the manufacturer's literature (Boehringer
30   Mannheim, Indianapolis, IN). One hundred ng of cleaved
and phosphatased pRmHa-3 vector is mixed with 300ng of
agarose gel purified Class I MHC heavy chain cDNA or β-2
microglobulin cDNA and ligated using T4 DNA ligase and
One Phor all buffer as described in the manufacturers'
35   literature. After incubation at 16 °C for five hours,

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the ligation mixture is used to transform competent *E. coli* JM83 (Maniatis, et al., *supra* (1982)).

Methods disclosed in Maniatis, et al., *supra* are used to prepare the cDNA needed. The presence of the

5   MHC heavy chain cDNA and its orientation in the vector is determined by restriction mapping. Bacteria containing the vector with the cDNA in the correct orientation relative to the metallothionein promoter are used for large scale preparation of DNA using the alkaline lysis

10   method and cesium chloride gradient purification. The amount of DNA obtained is determined spectrophotometrically.

D.   Transfection and Labeling of S2 Cells

S2 cells are grown in Schneider medium

15   (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal calf serum (heat treated for one hour at 55 °C), 100 units/ml penicillin, 100mg/ml streptomycin, and 1mM glutamine. (For convenience, this supplemented medium is hereinafter referred to as Schneider medium.) Cells are

20   grown at 27 °C and typically passaged every seven days by diluting 1:17 in fresh medium. Cells are converted to growth in serum free media (Excell 400 or 401 supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 1mM glutamine, and 500μg/ml G418 (JRH

25   Biosciences, Lenexa, KS) by initial dilution at 50% Schneider/50% Excell 401. One week later, cells may be passaged into 10% Schneider medium/90% Excell 401 and one week later into 100% Excell 401. Cells are maintained in this medium and passaged every seven days by diluting

30   1:17 in fresh medium.

15 X 10 S2 cells at a concentration of 10' cells per ml are plated out in 85mm petri dishes. Twelve hours later, calcium phosphate/DNA precipitates, prepared as described below (1 ml) are added dropwise to the

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removed and the cells transferred to a 175cm^2 flask in a
total volume of 50 ml in Schneider medium containing
500μg/ml Geneticin (G418) (Gibco/BRL, Grand Island, NY).
After 21 days, 20 ml of the culture is removed to a fresh
5   flask containing 30 ml of Schneider medium containing
500μg/ml G418. Ten days later, a stable population of
cells that weakly adhered to the flask and grew with a
doubling time of approximately 24 hours is obtained and
these cells are subsequently cultured and passaged in the
10   selection media as described above. Frozen aliquots of
these cells are prepared by collecting 5-20 X 10^6 cells
by centrifugation and resuspending them in 1 ml of cell
freezing media (93% fetal calf serum/7%
dimethylsulfoxide). Aliquots are then placed at -70 °C
15   for one week and subsequently transferred to liquid
nitrogen storage.

         Calcium phosphate precipitates are prepared as
described by Paabo, et al. (EMBO J. 5: 1921-27 (1986)),
except that 25μg of DNA is used per transfection. The
20   following combinations of DNA are used to prepare the
indicated transfectant:

(a)   MHC Class I heavy chain alone: 23μg heavy
chain expression vector DNA + 2μg of phshsneo DNA.

(b)   MHC Class I heavy chain + β-2 microglobulin:
25   11.5μg heavy chain expression vector DNA + 11.5μg of β-2
microglobulin (human or mouse) expression vector DNA +
2μg of phshsneo DNA.

         Other combinations of mouse genes are presented
in Table 1.

30   Twenty-four hours prior to metabolic labeling,
cells are plated out at a cell density of 3-5 X 10$^\cdot$
cells/ml (10 ml/85mm petri dish) in Schneider medium
containing 1mM CuSO$_4$. Thirty minutes prior to labelling
the medium is aspirated from the dishes and the cells are
35   washed with 2 X 10 ml of PBS and then incubated in Graces

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insect medium minus methionine and cysteine (special
order from Gibco/BRL, Grand Island, NY) for 20 minutes,
and then in 1 ml of this medium containing 0.1mCi ^{35}S
Trans label (New England Nuclear; duPont, Boston, MA).
5   After the labelling period, the labelling solution is
aspirated and the cells are either lysed immediately on
ice, with ice cold PBS/1% Triton X100 (1 ml) or after a
chase period in the presence of methionine containing
Schneider or Excell 400 medium (5 ml) (JRH Biosciences).
10 The chase medium is collected if soluble Class I MHC
molecules are being analyzed.

        The following operations are all carried out
with the lysates kept cold (less than 8 °C). The lysates
were collected into Eppendorf tubes, centrifuged in a
15 microfuge tube for 15 minutes at 13,000 X g, transferred
to a fresh tube containing 100μl of a 10% slurry of
protein A sepharose and placed on an end-over-end rotator
for two hours. Following a further centrifugation in the
microfuge for 15 minutes, the cell lysates are ready for
20 analysis.

        In experiments utilizing murine MHC, S2 cells
were transfected with the murine MHC recombinants
described above using the CaPO$_4$ precipitation method;
each heavy chain is transfected either alone or as a
25 50:50 mix with the vector encoding β-2 microglobulin. A
plasmid encoding neomycin resistance, phshsneo DNA, is
included in each transfection such that a population of
cells that stably expressed MHC Class I could be obtained
by growing the transfectants in selection medium
30 (Geneticin G418-sulphate, Gibco/BRL, Grand Island, NY).
        E.   Peptide Generation
        Antigenic peptides according to the present
invention may be obtained from naturally-occurring
sources or may be synthesized using known methods. In

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synthesized on an Applied Biosystems synthesizer, ABI
431A (Foster City, CA) and subsequently purified by HPLC.

   Isolation or synthesis of "random" peptides may
also be appropriate, particularly when one is attempting
5   to ascertain a particular epitope in order to load an
empty MHC molecule with a peptide most likely to
stimulate precursor CD8⁺ cells. One may produce a
mixture of "random" peptides via use of proteasomes (see,
e.g., Example 2.B.6) or by subjecting a protein or
10 polypeptide to a degradative process -- e.g., digestion
with chymotrypsin -- or peptides may be synthesized.
While we have observed that the cell lines of the present
invention are able to degrade proteins and polypeptides
into smaller peptides capable of being loaded onto human
15 Class I MHC molecules, it is preferable to introduce
smaller peptides -- e.g., 8-mers and 9-mers -- directly
into the cell culture to facilitate a more rapid loading
and expression process.

   If one is synthesizing peptides, e.g., random
20 8-, 9- and 1E-amino acid peptides, all varieties of amino
acids are preferably incorporated during each cycle of
the synthesis. It should be noted, however, that various
parameters --e.g.   such incompatibility of certain
amino acids -- result in a mixture which contains
25 peptides lacking certain amino acids. The process should
thus be adjusted as required -- i.e., by altering solvents
and reaction conditions -- to produce the greatest
variety of peptides.

   As noted hereinabove, murine heavy chains
30 complexed with human β-2 microglobulin were stable at
temperatures approximately 6-8 degrees higher than if
complexed with murine β2. It was also observed that the
stabilities imparted by peptide and xenogeneic β-2
microglobulin are additive. A large increase in the
35 thermostability of the Class I molecules occurs if 8-9

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mers are used, as compared to 12-25 mers; indeed, the difference between the stabilization imparted by the 8-9 mers compared with the larger peptides might be even greater than what was observed previously, for even

5   though the peptides have been purified by HPLC, it is likely that there is some contamination of the larger peptides by 8-9 mers.

The thermostability of a Class I molecule is apparently dependent on: (1) the origin of β-2

10   microglobulin; (2) the presence of peptide; and (3) the length and sequence of this peptide.

Previous work (U.S. Patent No. 5,314,813 to Peterson et al.; Jackson et al., PNAS USA 89: 12117-12121 (1992)) has shown that Class I MHC heavy chains can bind

15   peptide either alone or when they are associated with β-2 microglobulin. Surface expression of peptide-loaded human Class I MHC, however, appears to be best facilitated by loading the molecules with peptide after the heavy chains have complexed with β-2 microglobulin.

20   1. Expression of Human MHC

Once we determined that the thermostability of a Class I molecules is dependent on the origin of β-2 microglobulin, the presence of peptide, and the length and sequence of this peptide, we utilized this

25   information in the creation of cell lines capable of specifically activating CD8$^+$ cells via the expression of peptide-loaded human Class I MHC molecules.

Thermolability appears to be an inherent property of Class I molecules; it has presumably evolved

30   to ensure that Class I molecules which contain either no peptide or a peptide of poor binding properties (that confers little thermostability) self-destruct. In this way, the cell minimizes the number of empty Class I molecules on its surface, for such a situation would

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peptides could be bound and presented. Human Class I
molecules expressed in insect cells with human β2 are not
stable to extended incubation at 37 °C; neither are human
Class I molecules expressed in the mutant cell line T2
5   which has been shown to be deficient in peptide loading
onto the Class I molecules (Hosken and Bevan, Science
248: 367-70 (1990); Cerundolo, et al., Nature 345:
449-452 (1990)). Thus, it seems that the affinity between
the heavy chain and β-2 microglobulin has been carefully
10 conserved through co-evolution of the molecules such that
empty Class I molecules, or those carrying poorly-binding
peptides, self-destruct at the body temperature of the
"host" organism.
        Human Class I MHC molecules were expressed in
15 S2 cells. Cell lines co-expressing human β-2
microglobulin and HLA A2.2Y, HLA A2.1, HLA B7, or HLA B27
were established using previously-described methods.
Briefly, cDNAs encoding the above proteins were cloned
into the Drosophila expression vector pRmHa-3 and
20 cotransfected with a human β-2 microglobulin-containing
plasmid and phshsneo plasmid into S2 cells via methods
disclosed herein. Three to four weeks later, the
population of G418-resistanT-cells was diluted 1:5 with
fresh selection media. Once a healthy growing population
25 of cells was obtained, CuSO₄ was added to an aliquot of
cells and 24 hours later, cells were analyzed via flow
cytometry using a monoclonal antibody W6/32 (ATCC HB95,
Bethesda, MD) which recognizes a monomorphic determinant
of human Class I heavy chains when they are in
30 association with β-2 microglobulin. (See Barnstable, et
al., Cell 14: 9 (1978).) High levels of surface
expression of each of the human Class I molecules were
induced by the addition of CuSO₄ (data not shown). These
stable populations were sorted for high expressing cells
using cytofluorimetry as described below. It is these

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sorted populations of cells which were used for all
subsequent experiments.

    Twenty-four hours prior to FACS analysis, CuSO$_4$
is added to the stably transfected S2 cells (3-4 X 10^6
5   cells/ml) to a final concentration of 1mM, thereby
"switching on" expression from the transfected genes.
Cells are plated out in 24-well cluster dishes (2 ml per
well). Eight hours prior to FACS analysis, the CuSO$_4$
medium is replaced with fresh medium (1 ml) with or
10   without peptide at a concentration of 50μg/ml. 37 °C
temperature challenges are carried out by transferring
the dishes onto a flat surface in a 37 °C room at various
time intervals prior to harvesting the cells for
analysis.

15   To analyze surface expression of Class I MHC on
the S2 cells, aliquots of cells (5 X 10^5) are transferred
into tubes on ice, collected by centrifugation (1,000 X g
for 4 minutes), resuspended in 3 ml of PBS/1% BSA, 0.02%
sodium azide, collected by centrifugation and resuspended
20   in PBS/BSA (0.5 ml) containing the appropriate primary
antibody (ascites fluids Y3, 28:14:8S, 30.5.7, W6/32,
diluted 1:200). Rabbit antisera are diluted 1:500 and
B22.293 hybridoma supernatant is used directly. After a
one hour incubation on ice, cells are washed twice in 3
25   ml of PBS/BSA and resuspended in 0.5 ml of PBS/BSA
containing FITC conjugated secondary antibody (Cappell,
Durham, NC) and 1 μg r. propidium iodide. After a 30
minute incubation on ice, cells are washed once with
PBS/BSA and resuspended in this buffer at a concentration
30   of 1 X 10^7/ml. Samples are then analyzed by FACS 440
(Becton Dickinson. Dead cells stained with propidium
iodide, are excluded by including a live gate in the
analysis.

    For cell sorting, the same procedure outlined

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carried out in a sterile hood. Solutions, including
antibodies, are filter-sterilized, and Schneider media or
Excell 400 is used in place of PBS/BSA. Cells that
specifically bound the primary antibody are sorted using
5   a Becton Dickinson cell sorter. Sorted cells $(2-8 \times 10^5)$
are washed once in medium before plating out at a
concentration of 2×10^5 cells/ml.

       F.   <u>Loading of Membrane-Bound Empty MHC
           Molecules by <i>in vitro</i> Incubation with
10   Peptides</u>

          In order to demonstrate that the human Class I
molecules expressed on the surface of the *Drosophila*
cells were empty, the cells were incubated at 37 °C for
two hours and the cell surface expression was analyzed by
15   cytofluorimetry. The surface expression of both HLA B27
and A2.1 is greatly reduced if cells are incubated at
37 °C for 2 hours; however, preincubating the cells in
HIV peptides known to bind to the Class I molecules
affords significant thermal stability to the Class I,
20   while peptides that do not bind have little effect (see
Figure 4). (A 9-amino acid peptide ILKEPVHGV (SEQ ID
NO 42) from the POL protein of HIV binds and stabilizes
HLA A2.1. A nine-amino-acid peptide from the Vpr protein
of HIV binds and stabilizes B27 (FRIGCRHSR; SEQ ID
25   NO 41). These data show that the human Class I molecules
expressed on the surface of *Drosophila* cells are empty
and can be stabilized by binding specific HIV peptides.

          Figures 4 and 5 show peptide-induced
thermostabilization of HLA B27 and HLA A2.1 expressed on
30   the surface of *Drosophila* cells by HIV peptides.
Drosophila cells expressing either HLA B27 or A2.1 were
incubated with peptides where indicated and then either
maintained at 28 °C or incubated at 37 °C for two hours
prior to analysis of the surface expression of the
35   Class I molecules by use of the antibody W6/32 (from ATCC
_____) and cytofluorimetry. The mean fluorescence of each

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cell population is shown plotted against the incubation
conditions The HIV POL peptide (ILKEPVHGV, SEQ ID NO 42)
stabilizes A2.1 but not B27 (Figure 4), while the HIV Vpr
peptide (FRIGCRHSR, SEQ ID NO 41) stabilizes B27, but not
5   A2.1 (Figure 5).

Example 2
Preparation of Synthetic Antigen-Presenting Cells
A.   Osmotic Loading

Osmotic loading of SC2 and 3T3 cells with
10   ovalbumin protein was carried out as described by Moore,
et al., Cell 54: 777-785 (1988). The assay procedure is
as follows. In a 96-well dish, 1 X 10^5 Drosophila cells
(with or without peptide/protein loaded) or 3T3 cells
were cocultured with 1 X 10^5 B3/CD8 T-cell hybridoma
15   cells in 200μl of RPMI media supplemented with 10% fetal
bovine serum. After 24 hours of incubation, 100μl of the
supernatant from these cultures was added to 100μl of
RPMI containing 5,000 CTLL cells. The cells were
cocultured for 24 hours at 37 °C when 1μCi of 'H
20   thymidine (Amersham) was added. After a further
incubation of 15 hours at 37 °C, the incorporation of
radiolabel into the CTLL cells was determined by
scintillation counting.

Assays conducted with murine MHC also verified
25   that the insect cells are capable of loading peptide onto
the Class I molecules. Cells expressing as few as
200-500 MHC molecules containing a particular antigen can
be detected by a T-cell. As the Drosophila cells do not
accumulate chromium, an antigen presentation assay based
30   on B3/CD8, a T-cell hybridoma, was used. B3/CD8 is a
hybridoma between B3, cytotoxic T-cell specific for
ovalbumin peptide 253-276 presented by H-2K Class I
molecules, and CD8- bearing IL-2-secreting cell line (see
Carbone, et al., supra, 1989). Upon antigenic

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thymidine incorporation in IL-2-dependent T-cell line
CTLL (Gillis, et al., J. Immunol. 120: 2027 91978)).
Thus, by measuring the amount of IL-2 produced, one can
assay for T-cell recognition.

5   In order to provide an intracellular pool of
ovalbumin protein from which OVA peptides can be derived,
ovalbumin (Sigma Chem. Co., MO) was osmotically loaded
into the cells as described by Moore, et al, supra
(1988). Immediately after loading, the cells were mixed
10 with the T-cell hybridoma. After two days' incubation,
the medium was removed and assayed for IL-2. The amount
of IL-2 was determined by the ability of the medium to
support the growth of the IL-2-dependenT-cell line CTLL
(Gillis, et al., supra, 1978), and growth was quantitated
15 by the amount of radioactive thymidine incorporated into
the cells.

    S2 or 3T3 cells transfected with $K''/\beta 2$ were
incubated with ovalbumin protein (OvPro) or ovalbumin
peptide, OVA 24 (OvPep) in isotonic (Iso) or hypertonic
20 (Hyp) media. (Murine cell line BALB/3T3 is available
from the ATCC under accession number CCL 163.) After
treatment, cells were cocultured with the T-cell
hybridoma B3/CD8. B3/CD8 is a T-cell hybridoma between
B3 (Carbone, et al., J. Exp. Med. 169: 603-12 (1989)),
25 cytotoxic T-cell specific for ovalbumin peptide 253-276
presented by H-2K'' Class I molecules, and CD8- bearing
IL-2-secreting cell line. Upon antigenic stimulation,
B3/CD8 produces IL-2, measured by 'H thymidine
incorporation in IL-2-dependent cell line CTLL (Gillis,
30 et al., J. Immunol. 120: 2027 91978)). Thus, by
measuring the amount of IL-2 produced, one can assay for
T-cell recognition. The supernatant from the cocultures
were analyzed for IL-2 by 'H thymidine incorporation by
the IL-2-dependent cell line CTLL (ATCC No. TIB 214).

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The amount of ³H thymidine incorporated is plotted
against the initial cell treatments.

It can be seen in Figure 6 that the T-cells
responded well to the *Drosophila* cells if the ovalbumin
5   peptide was added to the culture medium, but no
recognition occurred if the cells were loaded with the
ovalbumin protein. The MHC Class I molecules expressed
on the cell surface of the insect cell are fully
functional in that they can bind peptide if it is added
10   to the culture medium and can present it in the correct
context for it to be recognized by a T-cell.

B.   Optimization of *In Vitro* Conditions

For the optimization of *in vitro* conditions for
the generation of specific cytotoxic T-cells, the culture
15   of *Drosophila* cell stimulator cells is preferably
maintained in serum-free medium (e.g. Excell 400).
Drosophila cell stimulator cells are preferably incubated
with >20µg/ml peptide. The effector:stimulator ratio
(lymphocyte:*Drosophila* cell ratio) is preferably in the
20   range of about 30:1 to 300:1. The maximum specific CD8⁺
is generally observed after five days of culture. The
culture of target cells for killing assay is preferably
maintained in a serum-free medium.

Example 3

25   Stimulation of Proliferation and Differentiation
of Armed Effector T-Cells

We have found that *Drosophila* S2 cells
transfected with MHC class I molecules and specific
assisting molecules are able to stimulate primary
30   responses from T-cells *in vitro*. We present data below
in this example from a mouse model system. In this
example, constructs coding for mouse MHC class I (L^d), β2
microglobulin, specific assisting molecules were used and
tested with CD8⁺ cells from lymph nodes of T-cell

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The data in Figure 7 provides evidence that the transfected *Drosophila* S2 cells express the protein products of the transfected murine genes. Flow cytometry using a fluorescence-activated cell sorter (FACS) and
5   fluorescently labelled antibodies were used to demonstrate the expression of L^d (MHC molecule which includes heavy chain and $\beta 2$) and the specific assisting molecules B7.1 (CD80) and ICAM-1 (CD54) molecules by transfected *Drosophila* S2 cells. Transfected cells were
10   separated with a FACS to obtain cells expressing L^d molecules and were then maintained *in vitro*.

The transfection of *Drosophila* S2 cells is summarized in Table 2. The data show L^d, B7.1 and ICAM-1 expression measured by flow cytometry on the cell lines
15   after induction with $CuSO_4$. It is apparent that, relative to the control antibody (ctr Ab), all of the transfectants express L^d molecules on the cell surface. Likewise, cells cotransfected with L^d and B7.1 ($L^d.B7$) express B7.1 but not ICAM-1, whereas cells cotransfected
20   with L^d and ICAM-1 ($L^d.ICAM$) express ICAM-1 but not B7.1; triple transfection with L^d, B7.1 and ICAM-1 ($L^d.B7.ICAM$) led to expression of all three molecules.

Using a standard tissue culture system (Cai, Z. and Sprent, J. (1994) J. Exp. Med. 179: 2005-2015), doses
25   of 5 x 10³ purified CD8⁺ 2C lymph node (LN) cells were cultured at 37 °C with doses of 3 x 10⁵ transfected fly cells ± peptides (10 μM final concentration). Peptides were synthesized by R. W. Johnson Pharmaceutical Research Institute (Sykulev, et al. (1994) Immunity 1: 15-22.
30   Proliferative responses were measured by adding ³HTdR (1 μCi/well) 8 hours prior to harvest. IL-2 production was measured by removing supernatants from the cultures at 48 hours and adding 50 μl supernatant to an IL-2 responsive indicator cell line (CTLL); proliferation of
35   the indicator line was measured by addition of ³HTdR.

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The data shown in Table 2 are the means of triplicate
cultures. The transfected *Drosophila* S2 cells die
rapidly at 37 °C and fail to incorporate ³HTdR at this
temperature.

5   The data in Table 2 demonstrate that the
transfectants are able to stimulate primary responses of
mouse T-cells.

         Table 2. Capacity of transfected fly cells to
stimulate primary proliferative responses and IL-2

10   production by CD8⁺ lymph node cells from 2C T-cell
receptor transgenic mice.

TABLE 2
³HTdR incorporation (cpm x 10³) with
transfected fly cells expressing:

Assay	Peptides added	L^d	L^d + B7.1	L^d + ICAM-1	L^d + B7.1 + ICAM-1	L^d + B7.1 combined with L^d + ICAM-1
Proliferation	-	0.2	0.1	0.3	0.2	-
(Day 3)	p2Ca	0.2	0.3	1.5	142.0	1.5
	QL9	0.2	60.9	73.9	263.7	132.9
IL-2 Production	-	0.3	0.2	0.1	1.2	-
(Day 2)	p2Ca	0.2	0.2	0.1	64.6	0.3
	QL9	0.1	0.4	0.2	158.6	0.5

The 2C T-cell receptor (TCR) is strongly reactive to L^d

25   molecules complexed with certain peptides, e.g. p2Ca (SEQ
ID NO 46) or QL9 (SEQ ID NO 47). These two peptides have
moderate to high affinity for soluble L^d molecules, 4 x
10⁷ M⁻¹ for p2Ca, and 4 x 10⁸ M⁻¹ for QL9 (Sykulev. *et
al.*). When complexed to soluble L^d molecules, the two

30   peptides also have high binding affinity for soluble 2C
TCR molecules. However, in both TCR binding and L^d
binding, the QL9 peptide clearly has a higher affinity

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Table 2 shows that proliferative responses and IL-2 production by the responder 2C cells to the weaker peptide, p2Ca, requires that the stimulator L^d-transfected cells coexpress both B7.1 and ICAM-1; a

5   mixture of cells expressing either L^d + B7.1 or L^d + ICAM-1 is nonstimulatory. By contrast, with the stronger peptide, QL9, L^d.fly cells expressing either B7 or ICAM elicit clearly-significant responses, although combined expression of B7 and ICAM generates much higher

10   responses. In contrast to these findings on T-cell proliferation, IL-2 production in response to the QL9 peptide requires joint expression of B7 and ICAM; expression of these molecules on separate cells is ineffective.

15   The results show that Drosophila cells transfected with murine class I molecules and costimulatory molecules induce murine T-cells to mount primary proliferative responses and lymphokine (IL-2) production in response to peptide antigens. The system

20   is also applicable to human T-cells and could be used to stimulate unprimed (or primed) T-cells specific for tumor-specific antigens in vitro; in vivo infusion of clonally-expanded T-cells specific for tumor-specific antigens might be therapeutic for patients with cancer.

25   Infusion of T-cells specific for viral antigens would be useful in patients with viral infections, e.g. HIV.

<u>Example 4</u>
<u>Immobilization of Biotinylated MHC Molecules</u>
<u>on Avidin-Coated Red Blood Cells</u>

30   NHS-LC-biotin, neutravidin and biotin-BMCC were purchased from Pierce (Rockford, IL). Sheep red blood cells were obtained from the Colorado Serum Company (Denver, CO). Drosophila S2 cells expressing L^d and recombinant L^d were prepared as described in Examples 1

35   and 2. Monoclonal antibodies 30.5.7 (anti-L^d) and 1B2

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(anti-clonotypic antibody to the 2C T-cell receptor) were used as hybridoma cell culture supernatants.

The protocol used is described by Muzykantov and Taylor (Anal. Biochem. (1994) 223, 142-148). Briefly, SRBC were washed 4 times in phosphate buffered saline (PBS), biotinylated using NHS-LC-biotin, washed again 4 times in PBS, incubated with neutavidin, and finally washed 4 times and stored at 4 °C in PBS containing 3% fetal calf serum and 0.02% sodium azide.

Recombinant L^d was biotinylated using biotin-BMCC, a maleimide-coupled biotin which reacts with thiol groups. L^d displays a free thiol group, the side chain of cystein 121, which is not in the peptide binding site. Biotinylation was performed as recommended by the manufacturer. Unreacted biotin was removed using Centricon 10.

Biotinylated L^d was immobilized by incubation at a final concentration of 0.2 mg/ml with avidin-coated SRBC for 30 minutes followed by washing in DMEM containing 10% fetal calf serum. SRBC with attached L^d were used immediately.

T-cells expressing the 2C TCR transgene from lymph nodes of mice were purified by magnetic depletion. Purified T-cells were consistently 97-98% positive for staining in flow cytofluorometry using the anti-clonotypic antibody 1B2.

Immobilization of biotinylated L^d on avidin-coated SRBC was done as indicated above. Attachment was assessed using flow cytofluorometry using anti-L^d antibody 30.5.7.

A typical experiment is represented in Figure 8. The negative control (cells minus antibody) is shown in dotted lines. The filled peak comprises cells labeled with fluorescent antibody. 99.78% of the cells

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range than the highest levels of intensity that we
observed for L^d on synthetic antigen-presenting cells.

K^b (MHC molecule which includes heavy chain and
$\beta 2$) was also biotinylated using the same procedure. We
5   could immobilize biotinylated K^b on avidin-coated SRBC as
assessed by flow cytofluorometry (Figure 9). 99.88% of
the cells were labeled.

Rosetting experiments verified that the
attached MHC molecules interacted functionally with
10   T-cells. *Drosophila* S2 cells expressing L^d, L^d-coated
SRBC were incubated with QL9 peptide (0.02 mM) or an
irrelevant peptide (MCMV, 0.02mM) for 30 minutes on ice;
2C+ T-cells were then added, the proportion being 10 2C+
T-cells for 1 *Drosophila* S2 cell, or 10 SRBC for 1 2C+
15   T-cell; the mixture was pelleted and kept on ice for at
least 30 min. Cells were then carefully resuspended and
rosettes were counted, a rosette being a *Drosophila* S2
cell bound to at least 2C+ T-cells, or a 2C+ T-cell
bound to at least FHC. Rosettes were observed in all
20   cases. Typically, of the lymphocytes were included
in rosettes when peptide was added. No rosette was
observed in the presence of the irrelevant peptide,
although occasional attachment of a few single cells was
observed.

25   These describe a new method to
immobilize high MHC class I molecules on
various surfaces cells, red blood cells, latex
beads) in native conformation as judged by monoclonal
antibody binding and rosetting experiments (T-cell
30   receptor binding. This method can be extended to other
synthetic surfaces including artificial phospholipid
membranes. Phosphatidylethanolamine as well as
avidin-coupled phospholipids are particularly relevant to
our studies. These phospholipids are commercially

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available from Lipex Biomembrane Inc., Vancouver, BC, Canada.

Example 5
Immobilization of Biotinylated MHC Molecules
on Avidin-Coated Latex Beads

5   Six micron diameter latex sulfate beads were purchased from Interfacial Dynamics Corporation (Portland, OR) and biotinylated according to the protocol described in Example 4.

10   Avidin-coated latex beads were prepared using a 1% suspension of the latex beads incubated in PBS containing 1mg/ml of neutravidin for one hour at room temperature. An equal volume of PBS containing 10% fetal calf serum was then added. After one hour of incubation

15   at room temperature, the beads were washed 3 times and used for binding of recombinant biotinylated L^d.

           Recombinant biotinylated L^d was immobilized by incubation at a final concentration of 0.2 mg/ml with avidin-coated latex beads for 30 minutes followed by

20   washing in DMEM containing 10% fetal calf serum. SRBC with attached L^d were used immediately.

           Rosetting experiments verified that the attached MHC molecules on latex beads interacted functionally with T-cells. *Drosophila* S2 cells

25   expressing recombinant L^d and L^d-coated latex beads were incubated with QL9 peptide (0.02 mM) or an irrelevant peptide (MCMV, 0.02mM) for 30 minutes on ice; 2C+ T-cells were then added, the proportion being 10 2C+ T-cells for 1 *Drosophila* S2 cell, or L^d-coated latex beads for 1 2C+

30   T-cell; the mixture was pelleted and kept on ice for at least 30 min. Cells were then carefully resuspended and rosettes were counted. a rosette being a *Drosophila* S2 cell bound to at least 3 2C+ T-cells, or a 2C+ T-cell bound to at least 3 latex beads. Rosettes were observed

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included in rosettes when QL9 peptide was added. No
rosette was observed in the presence of the irrelevant
peptide, although occasional attachment of a few single
cells was observed.

5   Example 6
            Immobilization and Detection of Recombinant
            Protein Bound to Various Solid Supports Such
                     as Plastic Microwell Plates
                The MHC molecules were immobilized by direct
10   binding to microtiter plates (Corning) and detected as
      follows:
                MHC K^h molecules were diluted to desired
      concentration in PBS, e.g. 0.001mg/ml for 100 ng /well.
      $100\mu l$ of diluted K^h was added to each well on the plastic
15   microtiter plate. The plate was incubated for 1 hour at
      room temperature. After incubation, the plate was washed
      once with PBS and $200\mu l$ 2% bovine serum albumin (BSA) in
      PBS + (.05%) and Tween (PBST) was added, and incubated
      for another hour at room temperature. The plate was
20   washed three times with PBST and biotinylated anti-K^{l} mAb
      was added (1:2500) in 2% BSA in PBS. The plate was
      incubated another hour at room temperature and washed
      three times with PBST. Avidin conjugated HRP was added
      (1:2500) in 2% BSA in PBS. Following another hour of
25   incubation at room temperature, the plate was washed
      three times with PBST and H_2O_2 or thophenyldiamine was
      added. The reaction was stopped with H_2SO_4. Reaction
      product was detected colorimetrically at 490 nm.
                Figure 10 shows the results of detecting the
30   presence of MHC K^{l} molecules using three different
      monoclonal antibodies.
                Recombinant MHC K^{l} molecules can alternatively
      be bound through biotin-avidin linked interactions with
      the substrate. In this embodiment, the microwell plates
35   were coated with $100\mu l$ avidin diluted in PBS to a

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concentration of 0.001mg/ml. Excess avidin was removed
by a PBS wash. The above procedure for presenting and
detecting K^b binding followed.

Recombinant MHC molecules may alternatively be
5   immobilized by a linkage based on a poly-histidine tag
added to the MHC interacting with the nickel bound to the
substrate.

The above procedure for binding and detection
is followed using nickel chelate coated microwell plates
10   (Xenopore) and recombinant MHC molecules with a
poly-histidine tag expressed using vector pRmHa/His$_6$
described above.

<u>Example 7</u>

<u>Direct Binding of Peptic to Soluble,</u>
15   <u>Empty Class I MHC Molecules</u> <u>*In Vitro*</u>

A.   Procedures

H-2K^b: prepared as described above in
Example 1.B.

H-2K^b Sol: K^b sol cDNA is a derivative of K^b,
20   encoding the extracellular portion of the Class I MHC
molecule. K^b sol cDNA may be produced by PCR according
to known methods, such as those described in Ennis, et
al., <u>PNAS USA</u> 87 (1990) and Zemmour, et al.,
<u>Immunogenetics</u> 33 (1991). Specifically, cDNA
25   encoding a truncated K^b molecule with a stop codon
inserted at the end of the alpha 3 domain at amino acid
position +275 is excised from the pCMU expression plasmid
as a Bam HI fragment and cloned into pRmHa-3 as K^b cDNA
The K^b sol cDNA is a derivative of the complete K^b cDNA
30   (see above) which is used as a template in a PCR reaction
using a 5' oligonucleotide that encompassed the Sty I
site, and the following 3' oligonucleotide:

5'   ATATGGATCCTCACCATCTCAGGGTGAGGGGC 3'

(SEQ ID NO 43)

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The resulting PCR fragment is blunt-end cloned into the
Sma I site of pBS (Stratagene, La Jolla, CA), sequenced,
and the remaining 5' sequence of K^b cloned into the Sty I
site. A cDNA encoding the complete K^bsol protein could
5 be obtained as a Bam HI restriction fragment.

    $H-2D^b$ and $H-2L^d$ are prepared as discussed in
Example 1.B. above.

    The cDNAs encoding K^b $\alpha1\alpha2\alpha3$ domains (274
residues) and murine β-2 microglobulin (99 residues) were
10 respectively cloned into the unique Bam HI site of an
expression vector harboring the metallothionein promoter
pRMHa-3 (Bunch, et al., <u>Nucleic Acid Res. 16</u>: 1043-1061
(1988)). <i>Drosophila</i> S2/M3 cells were transformed with
these recombinant plasmids in addition to plasmid
15 phshsneo (containing a neomycin-resistance gene) by the
calcium-phosphate precipitation method described
previously. The transformed cells selected against
neomycin-analog antibiotics G418 were grown at 27 °C in
serum-free medium and soluble heavy-chain K^b and β-2
20 microglobulin were co-expressed by the addition of 0.7mM
$CuSO_4$.

    The soluble, assembled heterodimer of K^b was
purified from the culture supernatants by affinity
chromatography using anti-K^b monoclonal antibody Y3,
25 followed by ion-exchange chromatography on a Pharmacia
Mono Q FPLC column according to the instructions of the
manufacturer (Pharmacia, Piscataway, NJ).
SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the
K^b preparation followed by staining with Coomassie blue
30 showed only one band of relative molecular mass (Mr) at
about 32,000 and one band of Mr at about 12,000 with no
detectable impurities. The highly-purified K^b was
dialyzed against phosphate-buffered saline (PBS),
filter-sterilized, and used for further study.

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Extinction coefficient of the soluble K^b ("K^bsol")
protein (43.2 kDa) is 69,200 $M^{-1}cm^{-1}$ at 280 nm.

      The purified K^b sol (0.3μM) in PBS with or
without 1% TX-100 were exposed to varying temperatures

5 (i.e., 4°, 23°, 32°, 37°, 42°, and 47°C) for one hour.
The proteins were then immunoprecipitated by incubating
with the monoclonal antibody Y3 and protein A sepharose
beads (Pharmacia, Piscataway, NJ) at 4 °C for two hours,
respectively. The samples were analyzed by 12.5%

10 SDS-PAGE, followed by staining with Coomassie blue. The
two thick bands on the gel are heavy and light chains of
antibody Y3. In another procedure, K^bsol (0.3μM) were
incubated with 50μM of peptides in PBS at 23 °C for two
hours to allow for K^bsol-peptide complex formation.

15 After the addition of 1% TX-100, the samples were exposed
to 12 °C, 37 °C, or 47 °C temperatures for one hour. The
complexes were immunoprecipitated and analyzed by
SDS-PAGE as described above. In a third procedure, K^bsol
(2.7μM) were incubated with 50μM of OVA-8, VSV-8 or SEV-9

20 peptides, respectively, at 23 °C for two hours. The
samples were applied on a 5% polyacrylamide IEF gel. IEF
was run from pH 5-7 and the gel was stained with silver.

      Next, VSV-8 peptide was radioiodinated using
the chloramine-T method (Hunter, et al., <u>Nature 194</u>:

25 495-6 (1962)) and free ^{125}I was removed by C_{18} column (OPC
cartridge, Applied Biosystems, Foster City, CA). The
labelled peptide was further purified by C_{18}
reverse-phase HPLC. After elution, the labelled peptide
was lyophilized and resuspended in PBS.

30   The specific activity of [^{125}I]VSV-8 (about 250
Ci/mmole) was determined spectrophotometrically by using
extinction coefficient of tyrosine at 274nm (1420
$M^{-1}cm^{-1}$). First, K^bsol (0.5μM) was mixed with [^{125}I]VSV-8
(1.5nM) and unlabelled VSV-8 (50nM) at 23 °C for 16 hours

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was analyzed by gel filtration (Superose 12, Pharmacia, Piscataway, NJ) in PBS. After elution, radioactivity contained in each fraction (0.05ml) was measured. Protein was monitored by absorbance at 280nm.

5   In a second procedure, [^{125}I]VSV-8 (0.39nM) was mixed with various concentrations of K^{b}sol in PBS containing 1% bovine serum albumin (BSA). After incubation at 23 °C for 2-16 hours, K^{b}sol-peptide complexes were separated from free peptide by small gel

10   filtration (Bio-Gel P30, BioRad, Richmond, CA) in PBS. P30 gel filtration permitted over 95% separation of bound and free peptide within about 5 minutes. Radioactivity of bound and free peptides was measured and the data were analyzed by linear regression. At maximal levels of

15   K^{b}sol offered, about 65% of the total labelled peptides were bound. This maximal binding capacity of labelled peptide to K^{b}sol protein deteriorated over time, presumably due to radiation by ^{125}I bound to VSV-8.

       In a third procedure, each sample contained

20   0.39nM of [^{125}I]VSV-8 (about 18,000 cpm), unlabelled peptides at the indicated concentration, and 30nM of K^{b}sol that gives about 50% of the [^{125}I]VSV-8 binding in the absence of unlabelled peptide at a final volume of 72μl. All components were dissolved and diluted in PBS

25   containing 1% BSA. After incubation for 2-16 hours at 23 °C, 50μl samples were analyzed by P30 gel filtration as described above. The dissociation constants for unlabelled peptides were determined from molar concentrations of [^{125}I]VSV-8 and unlabelled peptides

30   giving 50% inhibition of [^{125}I]VSV-8 binding to K^{b}sol as described. (See Muller, et al., Meth. Enzymol. 92: 589-601 (1983).)

       K^{b}sol (0.3μM) and [^{125}I]VSV-8 (0.39nM) were then incubated at 4° C, 23 °C, and 37 °C, and the association

35   was determined at various times by P30 gel filtration.

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Murine β-2 microglobulin was added, when necessary, before the incubation at the indicated concentration. The murine β-2 microglobulin was prepared by affinity chromatography using anti-β-2 microglobulin polyclonal

5 antibody K355 from culture supernatants of the recombinant *Drosophila* cells. (See also Logdberg, et al., Molec. Immun. 14: 577-587 (1979).) In another experiment, K^{b}sol (0.3μM or 1.8μM) and [^{125}I]VSV-8 (2.4nM) were incubated at 23 °C for two hours, and the

10 peptide-K^{b}sol complexes were isolated by P30 gel filtration. The samples contained very small amounts of [^{125}I]VSV-8 and K^{b}sol complexes (at the maximum, 2.4nM) and empty K^{b}sol at final concentration of about 50 to 300nM. To some samples, 3μM of β-2 microglobulin, 3μM of

15 β-2 microglobulin plus 20μM of unlabelled VSV-8, 20μM of unlabelled VSV-8, or 1% TX-100 were added. The samples were incubated for various times at 37 °C and the degree of dissociation was determined by passage over P30 columns.

20   B.   Discussion
               Class I MHC molecules present antigenic
peptides to cytotoxic T lymphocytes. Direct binding of peptide to Class I molecules *in vitro* has been hampered by either the presence of previously bound peptides at

25 the binding site (Chen and Perham, Nature 337: 743-5 (1989)) or the lack of binding specificity. (See, e.g., Frelinger, et al., J. Exp. Med. 172: 827-34 (1990); Choppin, et al., J. Exp. Med. 172: 889-99 (1990); Chen, et al., J. Exp. Med. 172: 931-6 (1990).) *In vitro*

30 analysis of peptide binding to soluble, empty Class I molecules purified from *Drosophila* cells transformed with truncated H-2K^{b}sol and murine β-2 microglobulin genes is disclosed herein. The results demonstrate that peptide binding is very rapid and naturally processed peptides

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213-6 (1990); Falk, et al., Nature 351: 290-6 (1991))
have the highest affinities to K^{h}sol of the nanomolar
range and indicate that K^{h}sol complexed with octapeptides
are stable, whereas those complexed with slightly shorter
5   or longer peptides are short-lived. Interactions between
free heavy chain and β-2 microglobulin is basically
reversible in the absence of detergent. Peptides
spontaneously bind to empty Class I molecules without
dissolution of β-2 microglobulin. However, excess β-2
10   microglobulin apparently promotes the binding of peptide
to empty Class I as a consequence of reassociation of
free heavy chain with β-2 microglobulin under conditions
where the heterodimers are unstable.

Soluble H-2K^h molecules (composed of the α1α2α3
15   domain of heavy chain) and murine β-2 microglobulin, were
purified from the culture supernatants of Drosophila
cells which were concomitantly transformed with the
truncated heavy chain and β-2 microglobulin genes.
Preliminary examinations suggested that Drosophila cells
20   express Class I MHC molecules devoid of endogenous
peptides on the cell surface. Some of the properties of
empty Class I molecules include the observation that they
are less stable at 37 °C and their structure is
stabilized by the binding of peptide. (See, e.g.,
25   Schumacher, et al., Cell 62: 563-7 (1990); Ljunggren, et
al., Nature 346: 476-80 (1990).) To confirm that
purified soluble K^h are also empty, their thermal
stability in detergent-free solution was examined.
Surprisingly, the proteins heated for one hour at 47 °C
30   were well recovered by immunoprecipitation using a
conformational antibody, Y3. This unexpected result led
us to add detergent, 1% Triton X-100 (polyoxyethylene (9)
octyl phenyl ether), to the protein solution, since
similar experiments to test the stability of Class I
35   molecules have always been conducted in detergent lysates

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(See Schumacher, et al., cited *supra*). The results
obtained in the presence of detergent show that the
purified K^{b}sol is now unstable at 37 °C. This and other
lines of evidence suggest that K^{b}sol heterodimer

5   disassembles into the heavy chain and β-2 microglobulin
at elevated temperatures and that detergent may prevent
β-2 microglobulin from reassociating with dissociated
free heavy chain (see below). Second, the possibility of
stabilizing purified K^{b}sol with peptides was studied.

10   The results of the first-described examination
demonstrated that the proteins can be stabilized only
when they are mixed with octapeptide (vesicular
stomatitis virus nucleocapsid protein [VSV-8], see
Table 3 below) which is shown to be a naturally processed

15   peptide (see Van Bleek, et al., cited *supra*). These
observations are consistent with the characteristics of
empty Class I molecules mentioned above.
         Independent support that the purified K^{b}sol
molecules are empty is provided by isoelectric focusing

20   (IEF) under native conditions (data not shown). The
soluble K^b purified from *Drosophila* cells exhibited a
much simpler pattern than HLA-A2 molecules purified from
human lymphoblastoid cell lines (see Figure 3 in Silver,
et al., Nature 350: 619-22 (1991)). The complicated

25   pattern of HLA-A2 on IEF is presumed to be the result of
the presence of heterogeneous peptides bound to the
molecules. The simple band of purified K^{b}sol indicates
the absence of endogenous peptides. In addition, the
incubation of K^{b}sol with antigenic peptides caused the

30   distinct shifts of band on IEF gel, reflecting the change
in isoelectric point of K^{b}sol due to the peptide binding.
It should be noted that such band-shifting was not
observed in HLA-A2 molecules when they were simply mixed
with peptides, unless HLA-A2 are incubated with peptides

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previously bound endogenous peptides. Taken together,
these observations on native IEF also indicate that
soluble K^b purified from *Drosophila* cells are empty.

The association of ^{125}I-labelled VSV-8 with
5   K^bsol was demonstrated by gel filtration (not shown).
The radioactivity of high molecular weight materials
corresponds to peptide-K^bsol complexes, while that of low
molecular weight materials represents free peptides.
Unlabelled VSV and ovalbumin (OVA) peptides could compete
10 with the labelled VSV-8 (see below), arguing that
$[^{125}I]$VSV-8 is bound specifically to K^bsol molecules.
Reversed-phase HPLC revealed that K^b-bound $[^{125}I]$VSV-8 has
the identical retention time to the input peptide. The
binding to K^bsol of the labelled VSV-8 was saturable,
15 exhibiting a dissociation constant (K_D) of about 33nM
(not shown). From the x-axis of the Scatchard plot, it
was noted that about 65% of the labelled VSV-8 is able to
bind to K^b.

To determine affinities of various peptides to
20 K^b, competitive radioimmunoassays (RIA) using $[^{125}I]$VSV-8
were carried out (data not shown). The inhibitory
peptides used for the RIA are listed in Table 3. K_r for
each peptide is summarized in Table 3 as well.

25   **TABLE 3**

Various Antigenic Peptides* Used in Present Studies

Code	Sequence	K_r (M)
VSV-7	GYVYQGL	5.3×10^{-8}
VSV-8	RGYVYQGL	3.7×10^{-9}
VSV-9N	LRGYVYQGL	7.3×10^{-8}
VSV-10N	DLRGYVYQGL	3.9×10^{-7}
VSV-9C	RGYVYQGLK	6.9×10^{-8}
VSV-10C	RGYVYQGLKS	2.1×10^{-6}
OVA-8	SIINFEKL	4.1×10^{-9}
OVA-9N	ESIINFEKL	8.9×10^{-8}

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OVA-10N	LESIINFEKL	2.8×10^{-7}
OVA-9C	SIINFEKLT	1.1×10^{-8}
OVA-10C	SIINFEKLTE	1.4×10^{-8}
OVA-24	EQLESIINFEKLTEWTSSNVMEER	7.1×10^{-5}
SEV-9	FAPGNYPAL	2.7×10^{-9}

VSV-8: Vesicular stomatitis virus nucleocapsid protein
52-59 (Van Bleek, et al., Nature 348: 213-216 (1990))
    OVA-8: Ovalbumin 257-264 (Carbone, et al., J. Exp.Med.
169: 603-12 (1989));
SEV-9: Sendai virus nucleoprotein 324-332 (Schumacher,
et al., Nature 350: 703-706 (1991))
* All peptides were purified by C_{18} reversed-phase HPLC
to exclude contaminating shorter peptides with different
binding properties. The 3-letter code designations and
SEQ ID NO for each peptide are given below.

VSV-7	GlyTyrValTyrGlnGlyLeu (SEQ ID NO 40, residue nos. 4-10)
VSV-8	ArgGlyTyrValTyrGlnGlyLeu (SEQ ID NO 40, residue nos. 3-10)
VSV-9N	LeuArgGlyTyrValTyrGlnGlyLeu (SEQ ID NO 40, residue nos. 2-10)
VSV-10N	AspLeuArgGlyTyrValTyrGlnGlyLeu (SEQ ID NO 40)
VSV-9C	ArgGlyTyrValTyrGlnGlyLeuLys (SEQ ID NO 44, residue nos. 1-9)
VSV-10C	ArgGlyTyrValTyrGlnGlyLeuLysSer (SEQ ID NO 44)
OVA-8	SerIleIleAsnPheGluLysLeu (SEQ ID NO 39, residue nos. 5-12)
OVA-9N	GluSerIleIleAsnPheGluLysLeu (SEQ ID NO 39, residue nos. 4-12)
OVA-10N	LeuGluSerIleIleAsnPheGluLysLeu (SEQ ID NO 39, residue nos. 3-12)
OVA-9C	SerIleIleAsnPheGluLysLeuThr (SEQ ID NO 39,

OVA-10C   SerIleIleAsnPheGluLysLeuThrGlu (SEQ ID NO 39,
           residue nos. 5-14)

OVA-24   GluGlnLeuGluSerIleIleAsnPheGluLysLeuThrGlu-TrpT
           hrSerSerAsnValMetGluGluArg (SEQ ID NO 39)

5   SEV-9   PheAlaProGlyAsnTyrProAlaLeu (SEQ ID NO 45)

The peptides of naturally processed size (8mer
for VSV and OVA, and 9mer for sendai virus nucleoprotein
[SEV]) had the highest and remarkably similar affinities
10   from the range of 2.7 to 4.1 nM. this exceedingly high
affinity of the natural peptides is consistent with
recent observations. (See, e.g., Schumacher, et al.,
Nature 350: 703-6 (1991); Christnick, et al., Nature 352:
67-70 (1991).) However, peptides that were shorter or
15   longer by as little as one or two residues lowered the
affinity by a factor of from 2 to 100. This reduction of
the affinity is even more drastic for a much longer
peptide; i.e., the affinity of 24mer peptide (OVA-24) is
more than 10,000 fold lower than that of OVA-8. These
20   results help to explain why earlier reports using longer
peptides claim the affinity of micromolar range. (See,
e.g., Frelinger, et al. and Choppin, et al., both cited
supra.) It is of particular interest that the extension
of peptides at the carboxyl terminus is much less
25   destructive of the affinity than extension at the amino
terminus. According to the three-dimensional structure
of HLA-A2, the peptide binding groove is formed by two
long α helices on the antiparallel β strands, and the
cleft is about 25 angstroms long, which is proposed to
30   accommodate an extended peptide chain of about eight
residues (see, e.g., Bjorkman, et al., Nature 329: 506-12
(1987)). At one end of the cleft, the α1 and α2 helices
come close together tightly, while at the other end, the
cleft is fairly open. It is now speculated that both VSV
35   and OVA peptide bind to the cleft in the same orientation

*and the carboxyl terminus of the peptides might interact with the relatively open end of the cleft so that the extension of peptide at the carboxyl terminus does not cause severe steric hindrance.

Examinations were then performed to determine the rate of peptide binding to K^b at 4 °C and 23 °C, respectively (not shown). Binding was very rapid, especially at 23 °C, with a half-time of about 5 minutes even in extremely low concentrations of labelled peptides (about 0.4nM). This contrasts with previous observations, which show a half-time of association of about two hours. (See, e.g., Choppin, et al., cited *supra*.) Again, only 65% of the total labelled peptide was able to bind. The addition of excess β-2 microglobulin did not affect the peptide-binding kinetics at such low temperatures that K^b heterodimer is stable (remained to be assembled). This implies that exchange of β-2 microglobulin is not a prerequisite for peptide binding; i.e., peptides can spontaneously bind to empty Class I molecules without dissociation of β-2 microglobulin. In contrast, excess free β-2 microglobulin apparently promotes peptide binding at 37 °C (data not shown). As the concentration of added β-2 microglobulin increased, more peptides bound to K^b molecules. Since empty K^b are unstable at 37 °C, a fraction of heterodimers must be dissociated to the heavy chain and β-2 microglobulin and thereby, the heterodimer must be in equilibrium with free heavy chain and free β-2 microglobulin. Then, the addition of β-2 microglobulin should shift the equilibrium toward the formation of heterodimer that can bind peptides. This view is supported by recent observations that there are substantial numbers of Class I free heavy chains on the normal cell surface and exogenously added β-2 microglobulin facilitates peptide binding to empty

Class I molecules on cells as a consequence of the reassociation of β-2 microglobulin with free heavy chain. (See, e.g., Rock, et al., Cell 65: 611-620 (1991); Kozlowski, et al., Nature 349: 74-77 (1991); Vitiello, et

5   al., Science 250: 1423-6 (1990).)

The dissociation kinetics of peptide at 37 °C were then observed. Immediately after isolating [^{125}I]VSV-8 and K^b complexes by gel filtration, the samples containing either 50 or 300 nM K^b were exposed to

10   37 °C temperatures. Some samples were supplemented with 3μM β-2 microglobulin and/or 20μM unlabelled VSV-8, or 1% TX-100. The dissociation of labelled peptides from K^h was measured at various times (not shown). In the presence of a large excess of unlabelled peptides, the

15   dissociation rate of peptide followed first-order kinetics with a half-time dissociation of about 36 minutes (a dissociation rate constant of 3.2 X 10^{-4} s^{-1}). This unexpected, relatively rapid dissociation of labelled peptide does not agree with some current views

20   of stable peptide-Class I complexes. In fact, the results ascertained (not shown) demonstrated that K^b and VSV-8 complexes are stable. This discrepancy must arise from the 10-fold lower affinity of radiolabelled VSV-8 (33 nM) compared with that of unlabelled VSV-8 (3.7 nM).

25   The first-order kinetics were also observed when the detergent was added instead of unlabelled peptide, indicating that the detergent makes the peptide dissociation process irreversible. In contrast, the peptide dissociation profile did not follow the

30   first-order kinetics in the absence of unlabelled peptide or the detergent. This suggests that the peptide association/dissociation is reversible and the binding of peptide is dependent on the concentration of heterodimer (compare the kinetics between 50 nM and 300 nM of K^b).

35   This became more evident when excess β-2 microglobulin

was added. These results support the previous argument
that interaction between the heavy chain, β-2
microglobulin and peptide are basically reversible at
37 °C, if not entirely, in the absence of detergent. It
5   is probable that a detergent such as TX-100 may prevent
β-2 microglobulin from reassociating with free heavy
chain at 37 °C. This could reasonably explain why Kᵇ
once heated to elevated temperatures in the absence of
detergent can be efficiently immunoprecipitated by
10 conformational antibody (not shown). Interestingly, the
addition of β-2 microglobulin did not suppress the
peptide dissociation in the presence of excess unlabelled
peptides, indicating that labelled peptides are released
from the complexes without dissociation of β-2
15 microglobulin. It should be remembered, however, that
the affinity of [³H]VSV-8 is about 10-fold lower than
that of the natural peptides. Therefore, this is not
necessarily the case for the natural peptides.

        The study using in vitro peptide-binding assay
20 systems suggests that peptide binding to Class I
molecules is a simple mass action and a ligand-receptor
interaction. The approach used herein allows
characterization of the peptide binding specificity to
Class I molecules and of the interaction of
25 peptide-Class I complexes with the T-cell receptor.

Example 8

Therapeutic Applications

A.   Class I Molecule Bank

        A reservoir or "bank" of insect cell lines may
30 be established and maintained, with each cell line
expressing one of the 50 to 100 most common Class I MHC
heavy chain, a β-microglobulin molecule, as well as at
least one assisting molecule. cDNAs encoding these
proteins may be cloned based on HLA variants obtained
35 from cell lines containing same -- e.g., via the

polymerase chain reaction (see Ennis, et al., PNAS USA
87: 2833-7 (1990)) -- and inserted into the appropriate
vector, such as an insect expression vector, to generate
cell lines expressing each HLA variant.

5   Testing according to the following protocol,
for example, can be used to determine which peptides
derived from the virus of choice bind the best to the
different Class I MHC molecules. The various cultures
may appropriately be labeled or catalogued to indicate
10   which Class I MHC molecules are best for use with
particular peptides. Alternatively, transient cultures
may be established as needed. As discussed herein, after
approximately 48 hours' incubation of a culture of insect
cells with a vector, that culture is apparently capable
15   of expressing empty MHC molecules which may be loaded
with the peptide(s) of choice for the purpose of
activating CD8· cells.

          B.   Preparation of "Special" Cell Lines
          After HLA typing, if insect cell lines
20   expressing the preferred HLA are not available, cDNAs
encoding the preferred HLA and assisting molecules may be
cloned via use of the polymerase chain reaction. The
primers disclosed in section B.1. above (SEQ ID NO 1
through SEQ ID NO 12) may be used to amplify the
25   appropriate HLA-A, -B, -C, -E, -F, or -G cDNAs in
separate reactions which may then be cloned and sequenced
as described in the methods disclosed in Example 1,
section 1 above. DNA is then purified from the PCR
reaction using a Gene Clean kit (Bio 101, San Diego, CA)
30   and ligated directly into the Sma I site of pRmHa-3.
Individual clones are isolated, the sequences verified,
and stable Drosophila cell lines expressing the HLA
established. Alternatively, a bulk population of
recombinant plasmids may be grown in large scale and DNA
35   purified by cesium chloride gradients. The purified DNA

is then used to transfect S2 cells using calcium
phosphate precipitation techniques. After 24 hours, the
precipitate is washed off the cells and replaced with
fresh Schneider media containing 1mM $CuSO_4$. Forty-eight
5 hours later, the bulk population of transiently
transfected cells is used for *in vitro* activation of CD8·
after incubation with syngeneic peptides or protease
digests of specific proteins.

Stable cell lines expressing the cloned HLA may
10 then be established. Alternatively, a population of
insect cells transiently expressing a bulk population of
cloned recombinant molecules from the PCR reaction may be
used for *in vitro* CD8· activation.

It is also possible to activate
15 haplotype-specific CD8s *in vitro* using insect cells
expressing Class I MHC incubated with peptides where the
cell line-expressed MHC is not the expressed element *in
vivo*. This provides a unique opportunity to proliferate
CD8· cells which recognize a specific antigen associated
20 with a particular MHC which would not be possible *in vivo*
due to allelic restriction. For example, a peptide (NP)
from the nuclear protein of Influenza virus is ordinarily
restricted to the D^b molecule; however, we have found
that such a peptide can bind to K^b (albeit more weakly
25 than to D^b) and can generate a degree of thermal
stability to the K^b (see Figure 3). Furthermore,
K^b-expressing *Drosophila* cells preincubated with the NP
peptide and cocultured with splenocytes from a B6 mouse
results in the *in vitro* activation of CD8· which
30 specifically recognize the K^b molecule associated with
the NP peptide. In addition, the reciprocal experiment
using a K^b-restricted peptide (OVA) derived from
ovalbumin and D^b-expressing *Drosophila* cells results in
the proliferation of CD8· which specifically recognize D^b
35 containing the OVA peptide. Such CD8s are able to kill

cells (EL4 OVA) transfected with cDNA encoding the ovalbumin protein, indicating that *in vivo*, some D^b molecules are loaded with the OVA peptide.

This system therefore provides a unique opportunity to proliferate CD8· against specific antigens presented by a Class I molecule which, *in vivo*, is not the restriction element for that peptide. Although enough antigen is presented *in vivo* by said Class I for the cell to be recognized by CD8· and killed, it is not enough to proliferate such CD8s *in vivo*. By loading empty Class I molecules expressed by *Drosophila* cells with peptide, we are able to override the *in vivo* restriction by providing an excess of antigenic peptide to the Class I molecule in a non-competitive environment such that enough antigen is presented by the Class I to activate the specific CD8· recognizing this complex.

C. AIDS Treatment

In vitro activated cells may be administered to patients for *in vivo* therapy. Preferably, the Class I MHC genotype (haplotype) of the individual is first determined. Conventional tissue typing is appropriate for this purpose and may be performed at the treatment center or by some appropriate commercial operation. Once the individual's HLA type(s) is (are) determined, the best combination of peptides and Class I MHC molecules suitable for the individual patient is ascertained and prepared as noted above and the appropriate insect cell lines and peptides are provided. Resting or precursor CD8· T-cells from the blood of the patient are then stimulated with the appropriate peptide-loaded MHC produced by the insect cell culture. After activation, the CD8· cells are reintroduced into the patient's bloodstream, and the disease process in the patient continues to be monitored. Methods of removing and re-introducing cellular components are known in the art

and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No. 4,690,915 to Rosenberg.

Additional treatments may be administered as necessary until the disease is sufficiently remediated. Similar treatment protocols are appropriate for use with other immunosuppressed individuals, including transplant patients, elderly patients, and the like.

D. Cancer Treatment

In cancer patients, a treatment procedure similar to that described above is utilized. However, in such patients, it should be anticipated that conventional therapy to reduce the tumor mass may precede the immune therapy described herein. Therefore, it is preferred that blood samples from the putative patient be obtained and stored (e.g. via freezing) prior to the commencement of conventional therapy such as radiation or chemotherapy, which tends to destroy immune cells. Since few, if any, forms of cancer arise in direct response to viral infection, target peptides for immune treatment are less readily observed. However, recent studies indicate that mutations in the oncogenes ras, neu, and p53 contribute to cancer in as much as 50% of all cancer cases. Thus, peptides derived from these mutated regions of the molecules are prime candidates as targets for the present therapy. Pursuant to the protocols disclosed herein, the best combination of peptides and Class I molecules for the individual patient may be determined and administered.

For example, many tumors express antigens that are recognized in vitro by CD8· cells derived from the affected individual. Such antigens which are not expressed in normal cells may thus be identified, as well as the HLA type that presents them to the CD8· cells, for precisely targeted immunotherapy using the methods of the

present invention. For example, van der Bruggen, et al. have described an antigen whose expression is directed by a specific gene and which antigen appears to be presented by HLA Al (Science 254: 1643-1647 (1991)). As various

5 human tumor antigens are isolated and described, they become good candidates for immunotherapeutic applications as described herein.

In another, alternative therapeutic mode, it may be feasible to administer the in vitro activated CD8·

10 cells of the present invention in conjunction with other immunogens. For example, the Large Multivalent Immunogen disclosed in U.S. Patent No. 5,045,320 may be administered in conjunction with activated CD8· cells.

It is also possible that cytokines such as IL-2

15 and IL-4, which mediate differentiation and activation of T-cells, may be administered as well, as cytokines are able to stimulate the T-cell response against tumor cells in vivo. It is believed that IL-2 plays a major role in the growth and differentiation of CD8· precursors and in

20 CD8· proliferation. The administration of IL-2 to cancer patients is frequently associated with an improved anti-tumor response which is likely related to induction of tumor-specific T-cells. However, the best therapeutic effects of IL-2 might be obtained by continuous local

25 rather than systemic administration of IL-2, thus minimizing the IL-2 toxicity and prolonging its biological activity. One may achieve local delivery via transfecting tumor cells with an IL-2 gene construct.

IL-2 cDNA is constructed as described by

30 Karasuyama and Melchers in Eur. J. Immunol. 18: 97-104 (1988). The complete cDNA sequence of IL-2 is obtained as an Xho I fragment from the plasmid pBMGneo IL-2 (see Karasuyama and Melchers, supra) and directly ligated into the Sal I site in pRmHa-3. Recombinant pRmHa-3 plasmid

35 with the insert in the correct orientation (determined

via restriction mapping with Hind III) is purified by cesium gradients and used to cotransfect S2 cells using the calcium phosphate technique. (A mixture of plasmid DNA was prepared for this purpose: 10μg pRmHa-3

5 containing IL-2 cDNA, 6μg each of pRmHa-3 plasmid containing MHC Class I heavy chain or β-2 microglobulin and 2μg of phshsneo DNA.) Stable cell lines which are inducible via CuSO₄ to express heavy chain, β-2 microglobulin and IL-2 were obtained by growing the

10 transfectants in G418 medium. These stable cell lines were coated with peptide and used in the *in vitro* assay as described above. Tumor cells transfected with IL-2 are observed to enhance the CTL (CD8) activity against the parental tumor cells and bypass CD4 and T helper

15 function in the induction of an antitumor or cytotoxic response *in vivo*. Therefore, increasing the potential of the *Drosophila* system via cotransfection with the IL-2 gene is suggested herein.

Example 9

20 ### Dose Dependence of the Production of Activated T-Cells Using the Antigen-Presenting System

Antigen-presenting cells (APC) were produced by transfecting *Drosophila* cells as described in Example 3 and then tested for their capacity to present antigen to

25 T-cells from the 2C line of transgenic mice. With mouse cells as antigen-presenting cells, this line displays strong alloreactivity for L^d molecules complexed with an endogenous 8-mer peptide, p2Ca (Leu-Ser-Pro-Phe-Pro-Phe-Asp-Leu, SEQ ID NO 46), derived from a Krebs cycle

30 enzyme, 2-oxoglutarate dehydrogenase (OGDH). The p2Ca peptide is exposed naturally bound to L^d on the surface of $H-2^d$ cells such as B10.D2 cells. The p2Ca peptide has intermediate binding affinity for soluble L^d molecules (4 x 10^6 M^{-1}) and high affinity for 2C TCR molecules (2 x 10^6

35 M^{-1} to 1 x 10^7 M^{-1}).

A closely-related 9-mer peptide, QL9 (Gln-Leu-Ser-Pro-Phe-Pro-Phe-Asp-Leu, SEQ ID NO 47), has even higher affinity for these molecules (2×10^8 M^{-1} for L^d and 2×10^7 M^{-1} for 2C TCR). Except for one additional

5   amino acid (glutamine) at the N terminus, QL9 has an identical sequence to p2Ca and, like p2Ca, forms part of the native sequence of OGDH.

With p2Ca and QL9 peptides (prepared in synthetic form), antigen-presenting cell requirements for

10   mature unprimed 2C CD8⁺ cells were studied *in vitro*. The responder CD8⁺ cells were purified from pooled lymph nodes (LN) of 2C mice on a C57BL/6 (B6, H-2^b) background by first removing CD4⁺ cells, class II-positive cells and B cells by mAb + complement treatment followed by

15   positive panning.

Cell suspensions were prepared from pooled cervical, axillary, inguinal and mesenteric LN of young adult mice using a tissue grinder. For cell purification, LN cells were first treated with a cocktail

20   of mAbs (anti-CD4, anti-HSA, anti-I-A^b) plus complement for 45 minutes at 37 °C. The surviving cells were further separated into CD8⁺ and CD8⁻ (CD4⁻) cells by panning at 4 °C for 60-90 minutes on petri dishes coated with anti-CD8 mAb. The attached CD8⁺ cells were

25   recovered by incubation at 37 °C for 5 minutes followed by vigorous pipetting. Non-attached cells were eluted and treated with anti-CD8 mAb and complement to obtain CD8⁻ 1B2⁻ 2C cells. More than 95% of the CD8⁺ cells obtained were clonotype-positive (IB2⁺) and 98% of these

30   cells displayed a naive (CD44^{lo}) phenotype.

TCR stimulation elicited a complex pattern of intracellular events which lead to early up-regulation of CD69 and CD25 (IL-2 receptor or IL-2R) on the cell surface. These changes are apparent within a few hours

35   of stimulation and are followed by cytokine synthesis and

cell proliferation. *Drosophila* cells were transfected with genes for L^d, L^d and B7.1 (L^d.B7), L^d and ICAM-1 (L^d.ICAM) or L^d, B7.1 and ICAM-1 (L^d.B7.ICAM). Figure 11 shows CD69 and CD25 expression on purified naive CD8⁻ 2C

5   cells stimulated for 12 hours with transfected *Drosophila* cells and p2Ca versus QL9 peptide at a concentration of 10 μM. Purified CD8⁻ 2C cells were incubated with various *Drosophila* cells plus a peptide (either p2Ca or QL9, 10 μM) in bulk (2 ml) culture for 12 hours and then

10   stained for CD69 or CD25. Either p2Ca or QL9 presented by *Drosophila* cells transfected with L^d.B7, L^d.ICAM or L^d.B7.ICAM were effective in stimulating the up-regulation of CD69 and CD25. However, non cultured 2C cells without either peptide or *Drosophila* cells did not

15   show up-regulation of CD69 and CD25 (top panel).

In the presence of *Drosophila* cells expressing L^d alone, induction of CD69 and CD25 expression on CD8⁻ 2C cells was low, but significant, with the strong QL9 peptide but barely detectable with the weaker p2Ca

20   peptide. With L^d.B7 or L^d.ICAM antigen-presenting cells, both peptides elicited marked up-regulation of CD69 and CD25. However, L^d.B7.ICAM antigen-presenting cells induced even higher expression of these molecules.

Drosophila cells transfected with L^d alone

25   failed to cause proliferation of 2C CD8⁻ cells to either p2Ca or QL9 peptide in the absence of exogenous lymphokines, consistent with the minimal capacity of these cells as antigen-presenting cells to induce CD69 and CD25 expression. The results are shown in Figure 12.

30   Responses to p2Ca (above) and QL9 (below) were measured by culturing 5×10^4 purified CD8⁻ 2C cells with 2×10^5 *Drosophila* cells in the presence or absence of the indicated concentrations of peptides for 3 days. [³H] TdR was added during the last 8 hours of culture; rIL-2

35   was added at a final concentration of 20 units/ml. The

data are the mean of triplicate cultures. When
supplemented with exogenous IL-2, however, both peptides
stimulated significant proliferative responses at high
doses (10 µM). The proliferative responses elicited by
5   QL9 were far stronger than for p2Ca (note the large
difference between the scales of x-axes of the upper and
the lower panel).

   Dose-response relationships for proliferation
on day 3 elicited by Drosophila cells transfected with
10   L^d.B7.ICAM and presenting p2Ca peptide were compared to
those elicited by Drosophila cells transfected with
L^d.B7, L^d.ICAM or L^d.B7.ICAM presenting QL9 peptide. The
results (means of triplicate cultures) are shown in
Figure 13. Drosophila APC (2 x 10^5 cells) were cultured
15   with 5 x 10^4 CD8$^+$ 2C cells for three days in the presence
or absence of peptides. [^{3}H] TdR was added during the
last 8 hours of culture; no IL-2 was added to the
cultures.

   Optimal proliferative responses elicited by
20   L^d.B7.ICAM antigen-presenting cells on day 3 required
high concentrations of p2Ca peptide, e.g. 10 µM (10^{-5} M),
(Figure 13). The results for QL9 peptide were different.
The does-response curves for L^d.B7 antigen-presenting
cells plus QL9 and L^d.ICAM antigen-presenting cells plus
25   QL9 approximated the results for L^d.B7.ICAM antigen-
presenting cells plus p2Ca (Figure 13). However, with
L^d.B7.ICAM antigen-presenting cells, proliferative
responses to QL9 peptide were maximal with 100 nM (10^{-7}
M) and were clearly apparent with doses as low as 10 pM
30   (10^{-11} M) (Figure 13. At high doses, e.g. 10 µM (10^{-5} M),
QL9 inhibited the proliferative response on day 3 (Figure
13, note the log scale.

   Inhibition of proliferation by L^d.B7.ICAM
antigen-presenting cells and QL9 peptide did not apply to
35   IL-2 production (Figure 14, right), and was only seen

with high doses of antigen-presenting cells (Figure 14, left). L^d.B7.ICAM antigen-presenting cells plus QL9 peptide was again found to be more effective in eliciting IL-2 production than L^s.B7.ICAM antigen-presenting cells

5   plus p2Ca peptide (Figure 14, right). The data indicates that antigen-presenting cells without peptides were ineffective in eliciting either response over the 100-fold range of antigen-presenting cells density tested (Figure 14, "-pep," open squares).

10   Changes in the dose-response relationships of the responses of CD8⁺ 2C cells on Day 3, Day 4 and Day 5 were examined using QL9 peptide with L^d.B7, L^d.ICAM or L^s.B7.ICAM antigen-presenting cells. The results are shown in Figure 15. Responses were measured with 5×10^4

15   CD8⁺ 2C cells and 3×10^3 antigen-presenting cells at the indicated peptide concentrations. The data are the mean results of triplicate cultures.

With an intermediate dose of 100 nM (10^{-7} M) QL9 peptide, proliferative responses to L^s.B7.ICAM

20   antigen-presenting cells were high on Day 3 (Figure 15, left), reached a peak on Day 4 (Figure 15, center) and then declined to low levels on Day 5 (Figure 15, right). With a high dose of 10 μM (10^{-5} M) QL9 peptide, however, the response was low on Day 3 (Figure 15, left), but then

25   increased markedly to reach a high peak on Day 5 (Figure 15, right).

Transient inhibition of proliferation induced by QL9 on Day 3 was only seen when the avidity of T-cell/APC interaction was very high. Decreasing the

30   avidity of T-cell/APC interaction by using lower doses of APC (Figure 14, left) or lower doses of QL9 peptide (Figure 15, left) augmented the Day 3 proliferative response. Reducing the avidity of T-cell/APC interaction enhanced the early (Day 3) proliferative response but

35   reduced the late (Day 5) response and also reduced IL-2

production (Figure 16, right). In Figure 16 the results
obtained using CD8⁺ 2C and CD8⁻ 2C cells on Days 2, 3, 4
and 5 are compared.

The observations apply with the highly
immunogenic L^d.B7.ICAM antigen-presenting cells.
However, with the less immunogenic L^d.B7 or L^d.ICAM
antigen-presenting cells, proliferative responses to QL9
peptide required high doses of peptide (Figure 13, Figure
15) and were crucially dependent upon CD8 expression by
the responder cells (Figure 16). These responses with
L^d.B7 and L^d.ICAM antigen-presenting cells reached a peak
on Days 3 or 4 (rather than Day 5) and were far lower
than with L^d.B7.ICAM antigen-presenting cells. With low
doses of QL9, e.g. 1 nM (10^{-9} M), proliferative responses
with L^d.B7 and L^d.ICAM antigen-presenting cells were
completely undetectable (< 100 cpm) (Figure 13). This
was in striking contrast to the results seen using
L^d.B7.ICAM antigen-presenting cells, where 1 nM QL9 led
to high responses (> 10,000 cpm) (Figure 13). In
contrast to the results with high doses of QL9 peptide
(10 μM, Table 2), the synergistic interaction between B7
and ICAM for proliferative responses became pronounced at
low peptide doses.

As can be seen, L^d.B7.ICAM cells act as
extremely potent antigen-presenting cells for naive 2C
cells. Raising the avidity of T-cell/APC interaction to
a high level inhibits the early proliferative response
but potentiates the late response and IL-2 production is
enhanced. For the high-affinity peptides such as QL9,
the synergy between B7 and ICAM is pronounced at low
doses of antigen.

Example 10
Production of Cytotoxic T-Cells
Using the Antigen Presenting System

Antigen-presenting cells (APC) were produced by
transfecting *Drosophila* cells as described in Example 3
and then tested for their ability to induce CTL activity.
CTL activity was tested on ^{51}Cr-labelled RMA.S-L^d targets
sensitized with QL9 peptide, no peptide or an irrelevant
peptide (MCMV). CD8$^+$ T cells (5 x 10^5) were cultured
with 2 x 10^6 transfected *Drosophila* cells in a volume of
2 ml in a 24-well culture plate. Cai, Z., and J. Sprent,
(1994). Peptides were present during the culture at a
concentration of 10 nM. After 4 days, the cells were
pooled and adjusted to the required number. To prepare
targets, RMA-S.L^c cells were labeled with ^{51}Cr (100
mCi/1-2 x 10^6 cells) at 37 °C for 90 minutes in the
presence or absence of peptides. After labeling, the
cells were thoroughly washed and resuspended in medium
with or without peptides. Specific ^{51}Cr release was
calculated according to established procedure. Id.

The capacity of L^d.B7, L^d.B7.ICAM and L^d.ICAM
antigen-presenting cells to induce CTL activity to 10 µM
QL9 peptide in cell cultures is shown in Figure 17.
Strongly immunogenic L^d.ICAM antigen-presenting cells
were efficient in generating QL9-specific CTL (Figure 17,
center). Significantly, L^d.B7 antigen-presenting cells
were also effective in generating CTL to QL9 (Figure 17,
left). The surprising finding, however, was that L^d.ICAM
antigen-presenting cells were totally unable to stimulate
CTL generation (Figure 17, right). This result
(representative of three different experiments) was
unexpected, because L^d.ICAM antigen-presenting cells were
no less efficient than L^d.B7 antigen-presenting cells at
inducing proliferative responses to QL9 (Figures 13 and
15). The L^d.ICAM-stimulated cultures in Figure 17

contained large numbers of blast cells, and total cell yields were about 3-fold higher than the input number.

The surprising finding that L^d.ICAM antigen-presenting cells were totally unable to stimulate CTL

5 generation applied to cultures not supplemented with exogenous lymphokines. However, when exogenous IL-2 was added to the cultures, L^d.ICAM antigen-presenting cells induced strong CTL activity to QL9 (Figure 18, right; 20u/ml exogenous IL-2).

10

Example 11
Proliferation of Normal T-Cells
Induced by the Antigen-Presenting System

Antigen-presenting cells (APC) were produced by

15 transfecting Drosophila cells as described in Example 3 and then tested for their ability to induce proliferation in normal (nontransgenic) murine T-cells.

The capacity of L^d.B7.ICAM Drosophila cells to induce a strong primary response of 2C.TCR transgenic

20 CD8⁻ cells raised the question whether Drosophila cells could act as antigen-presenting cells for normal (nontransgenic) CD8⁻ cells. Since the 2C mice were on a B6 ($H-2^b$) background, the response of normal B6 CD8⁻ cells was tested. Graded doses of CD8⁻ cells from normal

25 B6 mice were cultured with 10 μM QL9 peptide presented by L^d.B7.ICAM Drosophila antigen-presenting cells, i.e. a situation where a diverse repertoire of T-cells was exposed to only a single alloantigen (L^d + QL9) but at high concentration. As shown in Figure 19 (left),

30 presentation of QL9 by L^d.B7.ICAM antigen-presenting cells was indeed immunogenic for normal B6 CD8⁻ cells and led to appreciable proliferative responses on Day 3 (80,000 cpm) with large doses of responder cells (1 x 10⁶) in the absence of added cytokines; responses to an

35 unrelated peptide, MCMV, were much lower (though

significant) and no response occurred in the absence of
peptide. As expected, the response of normal B6 CD8⁻
cells to QL9 plus L^d.B7.ICAM antigen-presenting cells was
substantially lower than with normal B10.D2 spleen cells
5   as antigen-presenting cells (where the allostimulus was
provided by a multiplicity of self peptides bound to L^d,
K^d and D^d, but at low concentration) (Figure 19, right.
Note the difference in the Y axis scales).

10   Example 12
                    Activation Of Cytotoxic T-Cells Using
                Immobilized Purified Recombinant MHC Class I
                    Molecules And Assisting Molecules
         Except as noted, cells, materials and reagents
15   were prepared as described in Example 4. Biotinylated
anti-mouse CD28 monoclonal antibody (clone 37.51) was
purchased from Pharmingen (San Diego, CA). This antibody
is also available from Caltag (South San Francisco, CA).
This antibody, which binds to CD28 co-stimulatory
20   receptor, a ligand of B7.1 and B7.2 on the surface of
T-cells, augments the proliferation of T-cells (Gross et
al. J. Immunol. 149, 380-388, 1992). IL-2 was used as a
concanavalin A supernatant (10% final concentration).
         Substrates were prepared as follows: Fifty
25   microliters of PBS containing 1 microgram/ml of
neutravidin were added to each well of a 96 well cell
culture plate (Corning cat.#25860). After 2 hours at
room temperature, the wells were washed 3 times with PBS
prior to incubation with biotinylated molecules.
30   Avidin-coated mouse red blood cells were prepared as
described for avidin-coated sheep red blood cells in
Example 4. Avidin-coated latex beads were prepared as
described Example 4.
         Biotinylation of recombinant L^d was performed
35   as described in Example 4. Biotinylated recombinant L^d

was immobilized on the substrate together with
biotinylated anti-CD28 antibody. Avidin-coated red blood
cells or latex beads were incubated with 0.2 mg/ml
biotinylated L^d, 0.025 mg/ml biotinylated anti-CD28, or a
5   mixture thereof for 30 minutes at room temperature, then
washed 3 times in DMEM containing 10% FCS and used
immediately. Avidin-coated 96 well plates were incubated
with 50 microliters per well of 2 microgram/ml
biotinylated L^d, 0.25 microgram/ml anti-CD28, or mixtures
10   thereof for 30 minutes at room temperature, then washed 3
times using DMEM containing 10% FCS and used immediately.

       2C+ T-cells were prepared as described in
Example 4. CD8· cells from C57BL/6 mice were prepared
from the lymph nodes of these mice by magnetic depletion.
15   Purified cells were consistently 90-92% positive for CD8
expression, as assessed by flow cytofluorometry.

       T-cell activation was performed in culture
plates coated with purified L^d molecules and anti-CD28
antibody. T-cells and peptide (0.02 mM final
20   concentration) were added to 96 well plates coated with
L^d and/or anti-CD28, in a final volume of 0.2ml/well and
cultured for the appropriate time at 37 °C in humid
atmosphere containing 5% CO_2.

       T-cell activation using red blood cells or
25   latex beads coated with purified L^d molecules and anti-
CD28 antibody was performed in uncoated culture plates.
T-cells and peptide (0.02mM) were added to each well,
together with 100,000 red blood cells or latex beads.
Final volume was 0.2ml. Culture conditions were as
30   described above.

       T-cell mitogenesis was assayed by incorporation
of a pulse of 1 μCi of tritiated thymidine per well by
triplicate cell cultures. Cells were harvested 8 hours
later and thymidine incorporation was determined by
35   counting the filters in a scintillation counter.

RMA.S cells (target cells) expressing L^d were incubated with radiolabeled chromium for 90 minutes at 37 °C, washed 3 times and distributed in 96 well U-bottom plate (Costar cat#3799) (5000-10000 cells per well) in the presence of the appropriate peptide at 0.01mM. Various amounts of activated T-cells (effector cells) were added to reach effector/target ratios (E/T ratios) ranging between 150 and 1. After 5 hours of incubation at 37 °C, 0.1ml of supernatant was collected from each well and counted in a gamma counter. Percent lysis was determined by a standard method (Coligan et al., Current Protocols in Immunology, section 3.11, Wiley, New York (1991)).

Immobilized purified L^d and anti-CD28 antibody were mitogenic for 2C+ T-cells. When QL9 peptide was used, a thymidine incorporation above 100,000 cpm per well was consistently measured by day 3-5 of culture (Figure 20). Maximum thymidine incorporation in that same range was obtained using any of the three methods of activation (molecules immobilized on plastic, on red blood cells or on latex beads). When activation molecules were immobilized on plastic, L^d alone immobilized on plastic induced a transient mitogenesis (Figure 20, broken line) whereas L^d plus anti-CD28 antibody induced a higher and more sustained mitogenesis (Figure 20, solid line). Anti-CD28 antibody in addition to L^d was required for the induction of any mitogenesis in the model using red blood cells. However, anti-CD28 antibody alone did not induce any mitogenesis.

QL9-L^d complexes are recognized by the 2C T-cell receptor with a high affinity. Complexes of other peptides and L^d recognized by this receptor with a lower affinity were able to activate 2C+ T-cells; these included peptides p2Ca and SL9 (Figure 21). However, IL-2 added at day 2 of culture was required in order to

observe mitogenesis using these peptides. Activation was peptide specific since LCMV peptide, a control peptide that is not recognized by the 2C T-cell receptor, did not induce activation. Mitogenesis was measurable starting

5 with as little as 700 T-cells per well (96 plate well), demonstrating that the method activated a very small number of T-cells in a peptide specific manner.

2C+ T-cells were mixed with total CD8⁻ cells from C57BL/6 mice at a 1/99 ratio and cells were cultured

10 with immobilized L^d and anti-CD28 antibody in the presence of peptide QL9. IL-2 was added at day 2 of culture, and subsequently every other day. Cell activation and proliferation was noted, as evidenced by the formation of clumps, presence of enlarged cells that

15 progressively spread in the well. At day 12 of culture, cells were harvested and percentage of cells expressing the 2C T-cell receptor was assessed by staining with the anticlonotypic antibody 1B2 and analysis by flow cytofluorometry: 43% of the cells cultured initially in

20 the presence of peptide QL9 were 2C+, (Figure 22) whereas 49% of the cells expressed 2C after stimulation with peptide p2Ca (Figure 23), and 22% with peptide SL9 (Figure 24). This shows a considerable enrichment (43, 49 and 22 times) in specific T-cells after 12 days of

25 culture. Enrichment was observed even with SL9, a peptide that makes a low affinity complex with the 2C T-cell receptor. This method thus can specifically activate and enrich a small subpopulation of antigen specific T-cells out of a heterogenous mixture of T-

30 cells.

2C+ T-cells, activated using L^d and anti-CD28 antibody in the presence of QL9 peptide, were cultured for 5 days, then their cytotoxic capacity was assessed. The results, shown in Figure 25, demonstrated that

35 immobilized activation molecules induced the cells to

differentiate into effector cytotoxic T-cells. Lysis was specific, since it was observed in the presence of QL9 peptide but not in the presence of a control peptide (LCMV). Resting T-cells were thus activated using
5 immobilized molecules to differentiate into cytotoxic T-cells able to specifically kill targets.

As can be seen, immobilized purified MHC Class I and assisting molecules can be used to specifically activate naive resting T-cells into
10 cytotoxic T-cells. MHC class I and assisting molecules are sufficient for activation; no additional signal originating from antigen-presenting cells is necessary. MHC class I and assisting molecules immobilized on substrates, such as cell culture plates, provide an
15 appropriate tool for T-cell activation. Several advantages are offered by such coated substrates. They are easy to prepare and to manipulate, they can be coated with well-controlled amounts of molecules, ensuring reproducible activation conditions.
20

Example 13
Activation of Human Cytotoxic T-Cells

A unit of human blood (450 mls) collected in heparin (10 μ/ml) was obtained through the General
25 Clinical Research Center (GCRC) at Scripps Clinic, La Jolla, CA. The blood was first processed in a Ficoll-Hypaque density gradient in a 50 cc conical centrifuge (Histopaque 1077, Sigma) according to the manufacturer's specifications. Once the buffy coat was
30 obtained, the sample was washed in buffer 1 (D-PBS without Ca$^{..}$ or Mg$^{..}$), then buffer 2 (RPMI with 4% fetal calf serum (FCS)) and a final wash in buffer 3 (D-PBS + 1% human serum albumin (HSA, 25% Buminate/Baxter-Hyland) and 0.2% sodium citrate (w/v)).

The total peripheral blood mononuclear cell (PBMCs) preparation was counted from the washed cells. This preparation was then taken through a MaxSep™ isolation procedure (Baxter) where CD8⁺ cells were selected by negative selection. A cocktail of mAbs to cells targeted for removal (CD19-PharMingen, CD4-Ortho-mune, CD15-PharMingen, CD56-PharMingen, CD14-PRI) was prepared at 2 µg/ml of cells. The total PBMC cell count was diluted to 20 x 10⁶/ml in buffer 3 and the antibodies were added.

The mixture (approximately 40 mls) was rotated (4 rpm) on a rotary shaker at 4 °C, so that the tube mixed the sample end over end, for a total of 30 minutes. After the sensitization phase, the cells were washed with buffer 3 and resuspended in the same buffer with magnetic Dynal beads coated with sheep-anti-mouse IgG (SAM) (Dynabeads M450 #110.02). The stock of beads is usually 4 x 10⁸ beads/ml. The final bead:target cell ratio is 10:1.

To determine the final volume of beads to use, the formulas below were followed:

(Total cells sensitized) x (% of population of target cells) = Total theoretical target cell number.

Total theoretical target cell number x 10 = Total beads required.

Total beads required/Bead concentration of stock = Volume of beads required.

The volume of the final sensitized cells:beads mixture was approximately 50 mls. The mixture was put into a 150 ml Fenwal Transfer Pack (#4R2001), air added with a needle and the mixture rotating under similar conditions as above (30 minutes, 4 °C, 4 rpm, agitated end-over-end). At the end of the incubation period, the target cells were removed with a MaxSep™ separation device according to the manufacture's specifications.

The separated cells were transferred from the bag and counted to determine recovery. FACS analysis was performed to determine the purity of the sample.

The resulting separated human CD8⁺ cells were stimulated with *Drosophila* (fly) antigen-presenting cells which were transfected and shown to express human HLA A2.1, B7.1 and/or LFA-3. The fly cells were diluted to 10^6/ml in Schneider's medium with 10% FCS serum. On the following day CuSO4 was added to the cultured cells which were incubated for 24 hours at 27 °C and harvested. The harvested cells were washed and suspended in Insect X-press medium with a final peptide concentration of 100 µg/ml and incubated for 3 hours at 27 °C.

The peptides used were HIV-RT (ILKEPVHGV) (SEQ ID NO 48), Tyrosinase (YMNGTMSQV) (SEQ ID NO 49), and Influenza Matrix (GILGFVFTL) (SEQ ID NO 50).

The control peptide was derived from the core of the hepatitis virus and had the sequence FLPSDFFPSV (SEQ ID NO 51).

The fly antigen-presenting cells were added to the CD8⁺ cells at a ratio of 1:10 (APC:CD8⁺ T-cell). The cells were incubated in flat bottom wells (48 wells) for four days at 37 °C. At day 5, IL-2 (10 µ/ml) and IL-7 (10 µ/ml) (Genzyme) were added with a media change. On day 11 a CTL assay was performed.

JY, an EBV-transformed human B cell line expressing HLA 2.1, B7 was used as the target cell in the chromium release assay. Vissereu, M.J.W. et al. *J. Immunol.*, 154:3991-3998 (1995). JY cells were seeded at 3×10^5 cells/ml in RPMI with 10% FCS, 24 hours prior to the assay. The JY cells were counted and washed once in RPMI wash solution (4% Rehautin FCS, 1% HEPES, 0.025% gentamycin in RPMI) to provide 5×10^6 cells per sample. The cell pellet was gently resuspended in 100 µl of ^{51} Chromium stock (0.1 mCi, NEN) and placed in a 37 °C water

bath for 1 hour with agitation every fifteen minutes.
Labelled JY cells were washed four times for 6 minutes
each at 1400 rpm in 10 mls of RPMI wash solution. Cells
were adjusted to 1 x 10⁵ cells/ml in RPMI/10% Hyclone.
5   Efficiency of target cell labelling is confirmed by
standard gamma counter techniques.

        For peptide loading of target cells, two mls of
labelled JY cells (2 x 10⁵ cells) were incubated with
10 µg/ml peptide for 30 minutes at room temperature.
10   Peptide stock solutions (1mg/ml) were stored at -70 °C.
In a 96 well round bottom plate, 100 µl effector cells
and 100 µl peptide loaded target cells were combined at
ratios of 84:1, 17:1 and 3.4:1 (effector:target).
Controls to measure spontaneous release and maximum
15   release of ⁵¹Cr were included in duplicate. Samples were
incubated at 37 °C for 6 hours.

        K562 cells at a concentration of 10⁷/ml in RPMI
with 10% FCS were added at a ratio of 20:1 (unlabelled
K562:labelled JY). This erythroleukemic cell line was
20   used to reduce NK background cell lysis in the chromium
release assay. Plates were centrifuged at 1000 rpm for 5
minutes and 100 µl supernatant from each sample
transferred to 96 tube collection tubes. Analysis of cell
lysis is determined by standard gamma counting techniques
25   (Gammacell 1000, Nordion).

        CTL activity produced by activating human CD8⁺
T-cells with antigen-presenting cells loaded with
influenza matrix peptide (SEQ ID NO 50) is shown in
Figure 26. Prior exposure to influenza virus indicates
30   that this CTL activity was a secondary response. Data
points are the mean of values from triplicate cultures.
Antigen-presenting cells expressing A2.1, B7.1 and ICAM-1
were more effective than antigen-presenting cells
expressing A2.1 and B7.1 or antigen-presenting cells
35   expressing A2.1, B7.1 and LFA-3.

The results of activating human CD8⁺ T-cells with antigen-presenting cells loaded with HIV-RT peptide (SEQ ID NO 48) are presented in Figure 27. Since screening of the blood of this patient had indicated no

5   prior exposure to HIV, these results indicate the CTL activity was based on a primary response. In this case, only antigen-presenting cells expressing A2.1, B7.1 and ICAM-1 produced CTL activity that was significantly greater than control levels.

10   Figure 28 shows the CTL activity of human CD8⁺ T-cells activated by antigen-presenting cells loaded with tyrosinase peptide (SEQ ID NO 49). Tyrosinase is a normally occurring enzyme that is over-expressed in melanoma tumor cells. Again, the antigen-presenting

15   cells expressing all three molecules, ICAM-1 as well as A2.1 and B7.1, were most effective, especially at the lowest effector:target ratio tested.

These results show that the antigen presenting system is capable of producing effective CTL activity in

20   human CD8⁺ T-cells directed against a peptide that is derived from an endogenous protein that is over-expressed in tumor cells. This also demonstrates that this in vitro stimulation of CD8⁺ T-cells using both B7 and ICAM will generate cytotoxic CD8⁺ T-cells even against

25   peptides that would otherwise be recognized as self. This is contrary to present knowledge that such "self" peptides could not be used to create cytotoxic T-cells. This method greatly expands the number of possible tumor specific antigens that can be used to activate CD8⁺

30   T-cells against the tumor.

The foregoing is intended to be illustrative of the present invention, but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the

35   invention.

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCACCATGGC CGTCATGGCG CCC
23

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 23 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTCACACTT TACAAGCTCT GAG
23

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 23 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCACCATGCT GGTCATGGCG CCC
23

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 23 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

         (iii) HYPOTHETICAL: NO

         (iv) ANTI-SENSE: NO

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACTCGATG TGAGAGACAC ATC
23

(2) INFORMATION FOR SEQ ID NO:5:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 23 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

         (iii) HYPOTHETICAL: NO

         (iv) ANTI-SENSE: NO

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCACCATGCG GGTCATGGCG CCC
23

(2) INFORMATION FOR SEQ ID NO:6:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 23 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

         (iii) HYPOTHETICAL: NO

         (iv) ANTI-SENSE: NO

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTCAGGCTT TACAAGCGAT GAG
23

(2) INFORMATION FOR SEQ ID NO:7:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 23 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCACCATGCG GGTAGATGCC CTC
23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 23 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTTACAAGC TGTGAGACTC AGA
23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 23 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCACCATGGC GCCCCGAAGC CTC
23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 23 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGTCACACTT TATTAGCTGT GAG
23

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCACCATGGC GCCCCGAACC CTC
23

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTCACAATT TACAAGCCGA GAG
23

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 427 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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AATTCGTTGC AGGACAGGAT GTGGTGCCCG ATGTGACTAG CTCTTTGCTG CAGGCCGTCC
60

TATCCTCTGG TTCCGATAAG AGACCCAGAA CTCCGGCCCC CCACCGCCCA CCGCCACCCC
120

CATACATATG TGGTACGCAA GTAAGAGTGC CTGCGCATGC CCCATGTGCC CCACCAAGAG
180

TTTTGCATCC CATACAAGTC CCCAAAGTGG AGAACCGAAC CAATTCTTCG CGGGCAGAAC
240

AAAAGCTTCT GCACACGTCT CCACTCGAAT TTGGAGCCGG CCGGCGTGTG CAAAAGAGGT
300

GAATCGAACG AAAGACCCGT GTGTAAAGCC GCGTTTCCAA AATGTATAAA ACCGAGAGCA
360

TCTGGCCAAT GTGCATCAGT TGTGGTCAGC AGCAAAATCA AGTGAATCAT CTCAGTGCAA
420

CTAAAGG
427
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(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 740 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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ATTCGATGCA CACTCACATT CTTCTCCTAA TACGATAATA AAACTTTCCA TGAAAATAT
60

GGAAAAATAT ATGAAAATTG AGAAATCCAA AAAACTGATA AACGCTCTAC TTAATTAAAA
120

TAGATAAATG GGAGCGGCTG GAATGGCGGA GCATGACCAA GTTCCTCCGC CAATCAGTCG
180

TAAAACAGAA GTCGTGGAAA GCGGATAGAA AGAATGTTCG ATTTGACGGG CAAGCATGTC
240
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TGCTATGTGG CGGATTGCGG AGGAATTGCA CTGGAGACCA GCAAGGTTCT CATGACCAAG
300

AATATAGCGG TGTGAGTGAG CGGGAAGCTC GGTTTCTGTC CAGATCGAAC TCAAAACTAG
360

TCCAGCCAGT CGCTGTCGAA ACTAATTAAG TTAATGAGTT TTTCATGTTA GTTTCGCGCT
420

GAGCAACAAT TAAGTTTATG TTTCAGTTCG GCTTAGATTT CGCTGAAGGA CTTGCCACTT
480

TCAATCAATA CTTTAGAACA AAATCAAAAC TCATTCTAAT AGCTTGGTGT TCATCTTTTT
540

TTTTAATGAT AAGCATTTTG TCGTTTATAC TTTTTATATT TCGATATTAA ACCACCTATG
600

AAGTTCATTT TAATCGCCAG ATAAGCAATA TATTGTGTAA ATATTTGTAT TCTTTATCAG
660

GAAATTCAGG GAGACGGGGA AGTTACTATC TACTAAAAGC CAAACAATTT CTTACAGTTT
720

TACTCTCTCT ACTCTAGAGT
740

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 60 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTTGGATCC AGATCTACCA TGTCTCGCTT CGTGGCCTTA GCTGTGCTCG CGCTACTCTC
60

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGATCCGGAT GGTTACATGT CGCGATCCCA CTTAAC
36

(2) INFORMATION FOR SEQ ID NO:17:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 19 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

      (iii) HYPOTHETICAL: NO

       (iv) ANTI-SENSE: NO

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGAGCCGTGA CTGACTGAG
19

(2) INFORMATION FOR SEQ ID NO:18:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

      (iii) HYPOTHETICAL: NO

       (iv) ANTI-SENSE: NO

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCCTCGGCAC TGACTGACTC CTAG
24

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 38 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

      (iii) HYPOTHETICAL: NO

       (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GATCCTTATT AGATCTCACC ATCACCATCA CCATTGAG
38

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCGACTCAAT GGTGATGGTG ATGGTGAGAT CTAATAAG
38

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3875 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT   60

CTGGTTCCGA TAAGAGACCC AGAACTCCGG CCCCCCACCG CCCACCGCCA CCCCCATACA   120

TATGTGGTAC GCAAGTAAGA GTGCCTGCGC ATGCCCCATG TGCCCCACCA AGAGcTTTGC   180

ATCCCATACA AGTCCCCAAA GTGGAGAACC GAACCAATTC TTCGCGGGCA GAACAAAAGC   240

TTCTGCACAC GTCTCCACTC GAATTTGGAG CCGGCCGGCG TGTGCAAAAG AGGTGAATCG   300

AACGAAAGAC CCGTGTGTAA AGCCGCGTTT CCAAAATGTA TAAAACCGAG AGCATCTGGC   360

CAATGTGCAT CAGTTGTGGT CAGCAGCAAA ATCAAGTGAA TCATCTCAGT GCAACTAAAG   420

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GGGAATTCGA GCTCGGTACC CGGGGATCCT TATTAGATCT CACCATCACC ATCACCATTG    480

AGTCGACCTG CAGGCATGCA AGCTATTCGA TGCACACTCA CATTCTTCTC CTAATACGAT    540

AATAAAACTT TCCATGAAAA ATATGGAAAA ATATATGAAA ATTGAGAAAT CCAAAAAACT    600

GATAAACGCT CTACTTAATT AAAATAGATA AATGGGAGCG GCAGGAATGG CGGAGCATGG    660

CCAAGTTCCT CCGCCAATCA GTCGTAAAAC AGAAGTCGTG GAAAGCGGAT AGAAAGAATG    720

TTCGATTTGA CGGGCAAGCA TGTCTGCTAT GTGGCGGATT GCGGAGGAAT TGCACTGGAG    780

ACCAGCAAGG TTCTCATGAC CAAGAATATA GCGGTGAGTG AGCGGGAAGC TCGGTTTCTG    840

TCCAGATCGA ACTCAAAACT AGTCCAGCCA GTCGCTGTCG AAACTAATTA AGTCAATGAG    900

TTTTTCATGT TAGTTTCGCG CTGAGCAACA ATTAAGTTTA TGTTTCAGTT CGGCTTAGAT    960

TTCGCTGAAG GACTTGCCAC TTTCAATCAA TACTTTAGAA CAAAATCAAA ACTCATTCTA   1020

ATAGCTTGGT GTTCATCTTT TTTTTAATG ATAAGCATTT TGTCGTTTAT ACTTTTTATA   1080

TTTCGATATT AAACCACCTA TGAAGTCTAT TTTAATCGCC AGATAAGCAA TATATTGTGT   1140

AAATATTTGT ATTCTTTATC AGGAAATTCA GGGAGACGGG AAGTTACTAT CTACTAAAAG   1200

CCAAACAATT TCTTACAGTT TTACTCTCTC TACTCTAGAG TAGCTTGGCA CTGGCCGTCG   1260

TTTTACAACG TCGTGACTGG GAAAACCCTG GCGTTACCCA ACTTAATCGC CTTGCAGCAC   1320

ATCCCCCTTT CGCCAGCTGG CGTAATAGCG AAGAGGCCCG CACCGATCGC CCTTCCCAAC   1380

AGTTGCGCAG CCTGAATGGC GAATGGCGCC TGATGCGGTA TTTTCTCCTT ACGCATCTGT   1440

GCGGTATTTC ACACCGCATA TGGTGCACTC TCAGTACAAT CTGCTCTGAT GCCGCATAGT   1500

TAAGCCAGCC CCGACACCCG CCAACACCCG CTGACGCGCC CTGACGGGCT TGTCTGCTCC   1560

CGGCATCCGC TTACAGACAA GCTGTGACCG TCTCCGGGAG CTGCATGTGT CAGAGGTTTT   1620

CACCGTCATC ACCGAAACGC GCGAGACGAA AGGGCCTCGT GATACGCCTA TTTTTATAGG   1680

TTAATGTCAT GATAATAATG GTTTCTTAGA CGTCAGGTGG CACTTTTCGG GGAAATGTGC   1740

GCGGAACCCC TATTTGTTTA TTTTTCTAAA TACATTCAAA TATGTATCCG CTCATGAGAC   1800

AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA GAGTATGAGT ATTCAACATT   1860

TCCGTGTCGC CCTTATTCCC TTTTTTGCGG CATTTTGCCT TCCTGTTTTT GCTCACCCAG   1920

AAACGCTGGT GAAAGTAAAA GATGCTGAAG ATCAGTTGGG TGCACGAGTG GGTTACATCG   1980

AACTGGATCT CAACAGCGGT AAGATCCTTG AGAGTTTTCG CCCCGAAGAA CGTTTTCCAA   2040

TGATGAGCAC TTTTAAAGTT CTGCTATGTG GCGCGGTATT ATCCCGTATT GACGCCGGGC   2100

AAGAGCAACT CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTGAG TACTCACCAG   2160

TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA ATTATGCAGT GCTGCCATAA   2220

CCATGAGTGA TAACACTGCG GCCAACTTAC TTCTGACAAC GATCGGAGGA CCGAAGGAGC   2280

TAACCGCTTT TTTGCACAAC ATGGGGGATC ATGTAACTCG CCTTGATCGT TGGGAACCGG   2340
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AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC GATGCCTGTA GCAATGGCAA   2400

CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCGG CAACAATTAA   2460

TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT GCGCTCGGCC CTTCCGGCTG   2520

GCTGGTTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG GTCTCGCGGT ATCATTGCAG   2580

CACTGGGGCC AGATGGTAAG CCCTCCCGTA TCGTAGTTAT CTACACGACG GGGAGTCAGG   2640

CAACTATGGA TGAACGAAAT AGACAGATCG CTGAGATAGG TGCCTCACTG ATTAAGCATT   2700

GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA CTTCATTTTT   2760

AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA ATCCCTTAAC   2820

GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA GATCAAAGGA TCTTCTTGAG   2880

ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT TGCAAACAAA AAAACCACCG CTACCAGCGG   2940

TGGTTTGTTT GCCGGATCAA GAGCTACCAA CTCTTTTTCC GAAGGTAACT GGCTTCAGCA   3000

GAGCGCAGAT ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA   3060

ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG GCTGCTGCCA   3120

GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG ATAGTTACCG GATAAGGCGC   3180

AGCGGTCGGG CTGAACGGGG GGTTCGTGCA CACAGCCCAG CTTGGAGCGA ACGACCTACA   3240

CCGAACTGAG ATACCTACAG CGTGAGCATT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA   3300

AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG AGGGAGCTTC   3360

CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC TGACTTGAGC   3420

GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG GAAAACGCC AGCAACGCGG   3480

CCTTTTTACG GTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTCTTCC TGCGTTATCC   3540

CCTGATTCTG TGGATAACCG TATTACCGCC TTTGAGTGAG CTGATACCGC TCGCCGCAGC   3600

CGAACCGACC GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG GAAGAGCGCC CAATACGCAA   3660

ACCGCCTCTC CCCGCGCGTT GGCCGATTCA TTAATGCAGC TGGCACGACA GGTTTCCCGA   3720

CTGGAAAGCG GGCAGTGAGC GCAACGCAAT TAATGTGAGT TAGCTCACTC ATTAGGCACC   3780

CCAGGCTTTA CACTTTATGC TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA   3840

ATTTCACACA GGAAACAGCT ATGACATGAT TACCG                              3875
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(2) INFORMATION FOR SEQ ID NO:22:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 71 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATCCTTATT AGATCTTACC CATACGACGT CCCAGATTAC GCTCGATCTC ACCATCACCA   60

TCACCATTGA G   71

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 71 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCGACTCAAT GGTGATGGTG ATGGTGAGAT CGAGCGTAAT CTGGGACGTC GTATGGGTAA   60

GATCTAATAA G   71

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3908 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT   60

CTGGTTCCGA TAAGAGACCC AGAACTCCGG CCCCCCACCG CCCACCGCCA CCCCCATACA   120

TATGTGGTAC GCAAGTAAGA GTGCCTGCGC ATGCCCCATG TGCCCCACCA AGAGcTTTGC   180

ATCCCATACA AGTCCCCAAA GTGGAGAACC GAACCAATTC TTCGCGGGCA GAACAAAAGC   240

TTCTGCACAC GTCTCCACTC GAATTTGGAG CCGGCCGGCG TGTGCAAAAG AGGTGAATCG   300

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AACGAAAGAC CCGTGTGTAA AGCCGCGTTT CCAAAATGTA TAAAACCGAG AGCATCTGGC    360

CAATGTGCAT CAGTTGTGGT CAGCAGCAAA ATCAAGTGAA TCATCTCAGT GCAACTAAAG    420

GGGAATTCGA GCTCGGTACC CGGGGATCCT TATTAGATCT TACCCATACG ACGTCCCAGA    480

TTACGCTCGA TCTCACCATC ACCATCACCA TTGAGTCGAC CTGCAGGCAT GCAAGCTATT    540

CGATGCACAC TCACATTCTT CTCCTAATAC GATAATAAAA CTTTCCATGA AAAATATGGA    600

AAAATATATG AAAATTGAGA AATCCAAAAA ACTGATAAAC GCTCTACTTA ATTAAAATAG    660

ATAAATGGGA GCGGCAGGAA TGGCGGAGCA TGGCCAAGTT CCTCCGCCAA TCAGTCGTAA    720

AACAGAAGTC GTGGAAAGCG GATAGAAAGA ATGTTCGATT TGACGGGCAA GCATGTCTGC    780

TATGTGGCGG ATTGCGGAGG AATTGCACTG GAGACCAGCA AGGTTCTCAT GACCAAGAAT    840

ATAGCGGTGA GTGAGCGGGA AGCTCGGTTT CTGTCCAGAT CGAACTCAAA ACTAGTCCAG    900

CCAGTCGCTG TCGAAACTAA TTAAGTAAT GAGTTTTTCA TGTTAGTTTC GCGCTGAGCA    960

ACAATTAAGT TTATGTTTCA GTTCGGCTTA GATTTCGCTG AAGGACTTGC CACTTTCAAT   1020

CAATACTTTA GAACAAAATC AAAATTCATT CTAATAGCTT GGTGTTCATC TTTTTTTTTA   1080

ATGATAAGCA TTTTGTCGTT TATACTTTTT ATATTTCGAT ATTAAACCAC CTATGAAGTc   1140

tATTTTAATC GCCAGATAAG CAATATATTG TGTAAATATT TGTATTCTTT ATCAGGAAAT   1200

TCAGGGAGAC GGGAAGTTAC TATTTACTAA AAGCCAAACA ATTTCTTACA GTTTTACTCT   1260

CTCTACTCTA GAGTAGCTTG GTATTGGTCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC   1320

CTGGCGTTAC CCAACTTAAT CGTTTTGTAG CACATCCCCC TTTCGCCAGC TGGCGTAATA   1380

GCGAAGAGGC CCGCACCGAT CGTTTTTTTT AACAGTTGCG CAGCCTGAAT GGCGAATGGC   1440

GCCTGATGCG GTATTTTCTC CTTATGTATT TGTGCGGTAT TTCACACCGC ATATGGTGCA   1500

CTCTCAGTAC AATCTGCTCT GATTTTTCTT ATTTAAGCCA GCCCCGACAC CCGCCAACAC   1560

CCGCTGACGC GCCCTGACGG GTTTTTTTTT TCCCGGCATC CGCTTACAGA CAAGCTGTGA   1620

CCGTCTCCGG GAGCTGCATG TGTTAAATTT TTTCACCGTC ATCACCGAAA CGCGCGAGAC   1680

GAAAGGGCCT CGTGATACGC CTATTTTTAT AGGTTAATGT CATGATAATA ATGGTTTCTT   1740

AGACGTCAGG TGGCACTTTT CGGGGAAATG TGCGCGGAAC CCCTATTTGT TTATTTTTCT   1800

AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG CTTCAATAAT   1860

ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT CGCCCTTATT CCCTTTTTTG   1920

CGGCATTTTG CCTTCCTGTT TTTGCTCACC CAGAAACGCT GGTGAAAGTA AAAGATGCTG   1980

AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA TCTCAACAGC GGTAAGATCC   2040

TTGAGAGTTT TCGCCCCGAA GAACGTTTTC CAATGATGAG CACTTTTAAA GTTCTGCTAT   2100

GTGGCGCGGT ATTATCCCGT ATTGACGCCG GGCAAGAGCA ACTCGGTCGC CGCATACACT   2160

ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA AAAGCATCTT ACGGATGGCA   2220
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TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG TGATAACACT GCGGCCAACT    2280

TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACCGC TTTTTTGCAC AACATGGGGG    2340

ATCATGTAAC TCGCCTTGAT CGTTGGGAAC CGGAGCTGAA TGAAGCCATA CCAAACGACG    2400

AGCGTGACAC CACGATGCCT GTAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG    2460

AACTACTTAC TCTAGCTTCC CGGCAACAAT TAATAGACTG GATGGAGGCG GATAAAGTTG    2520

CAGGACCACT TCTGCGCTCG GCCCTTCCGG CTGGCTGGTT TATTGCTGAT AAATCTGGAG    2580

CCGGTGAGCG TGGGTCTCGC GGTATCATTG CAGCACTGGG GCCAGATGGT AAGCCCTCCC    2640

GTATCGTAGT TATCTACACG ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA    2700

TCGCTGAGAT AGGTGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA GTTTACTCAT    2760

ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA AAGGATCTAG GTGAAGATCC    2820

TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT TTCGTTCCAC TGAGCGTCAG    2880

ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT TTTTCTGCGC GTAATCTGCT    2940

GCTTGCAAAC AAAAAAACCA CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC    3000

CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC    3060

TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT ACATACCTCG    3120

CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA TAAGTCGTGT CTTACCGGGT    3180

TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC GGGCTGAACG GGGGGTTCGT    3240

GCACACAGCC CAGCTTGGAG CGAACGACCT ACACCGAACT GAGATACCTA CAGCGTGAGC    3300

ATTGAGAAAG CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA    3360

GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG AAACGCCTGG TATCTTTATA    3420

GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT TTTGTGATGC TCGTCAGGGG    3480

GGCGGAGCCT ATGGAAAAAC GCCAGCAACG CGGCCTTTTT ACGGTCCTGG CCTTTTGCTG    3540

GCCTTTTGCT CACATGTCTT TCCTGCGTTA TCCCCTGATT CTGTGGATAA CCGTATTACC    3600

GCCTTTGAGT GAGCTGATAC CGCTCGCCGC AGCCGAACCG ACCGAGCGCA GCGAGTCAGT    3660

GAGCGAGGAA GCGGAAGAGC GCCCAATACG CAAACCGCCT CTCCCCGCGC GTTGGCCGAT    3720

TCATTAATGC AGCTGGCACG ACAGGTTTCC CGACTGGAAA GCGGGCAGTG AGCGCAACGC    3780

AATTAATGTG AGTTAGCTCA CTCATTAGGC ACCCCAGGCT TTACACTTTA TGCTTCCGGC    3840

TCGTATGTTG TGTGGAATTG TGAGCGGATA ACAATTTCAC ACAGGAAACA GCTATGACAT    3900

GATTACCG    3908
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(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 base pairs
        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GATCCTTATT AGATCTCACC ATCACCATCA CCATTGTTGA G   41

(2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TCGACTCAAC AATGGTGATG GTGATGGTGA GATCTAATAA G   41

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3878 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT   60

CTGGTTCCGA TAAGAGACCC AGAACTCCGG CCCCCCACCG CCCACCGCCA CCCCCATACA   120

TATGTGGTAC GCAAGTAAGA GTGCCTGCGC ATGCCCCATG TGCCCCACCA AGAGCTTTGC   180

ATCCCATACA AGTCCCCAAA GTGGAGAACC GAACCAATTC TTCGCGGGCA GAACAAAAGC   240

TTCTGCACAC GTCTCCACTC GAATTTGGAG CCGGCCGGCG TGTGCAAAAG AGGTGAATCG   300

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AACGAAAGAC CCGTGTGTAA AGCCGCGTTT CCAAAATGTA TAAAACCGAG AGCATCTGGC    360

CAATGTGCAT CAGTTGTGGT CAGCAGCAAA ATCAAGTGAA TCATCTCAGT GCAACTAAAG    420

GGGAATTCGA GCTCGGTACC CGGGGATCCT TATTAGATCT CACCATCACC ATCACCATTG    480

TTGAGTCGAC CTGCAGGCAT GCAAGCTATT CGATGCACAC TCACATTCTT CTCCTAATAC    540

GATAATAAAA CTTTCCATGA AAAATATGGA AAAATATATG AAAATTGAGA AATCCAAAAA    600

ACTGATAAAC GCTCTACTTA ATTAAAATAG ATAAATGGGA GCGGCAGGAA TGGCGGAGCA    660

TGGCCAAGTT CCTCCGCCAA TCAGTCGTAA AACAGAAGTC GTGGAAAGCG GATAGAAAGA    720

ATGTTCGATT TGACGGGCAA GCATGTCTGC TATGTGGCGG ATTGCGGAGG AATTGCACTG    780

GAGACCAGCA AGGTTCTCAT GACCAAGAAT ATAGCGGTGA GTGAGCGGGA AGCTCGGTTT    840

CTGTCCAGAT CGAACTCAAA ACTAGTCCAG CCAGTCGCTG TCGAAACTAA TTAAGTtAAT    900

GAGTTTTTCA TGTTAGTTTC GCGCTGAGCA ACAATTAAGT TTATGTTTCA GTTCGGCTTA    960

GATTTCGCTG AAGGACTTGC CACTTTCAAT CAATACTTTA GAACAAAATC AAAACTCATT   1020

CTAATAGCTT GGTGTTCATC TTTTTTTTTA ATGATAAGCA TTTTGTCGTT TATACTTTTT   1080

ATATTTCGAT ATTAAACCAC CTATGAAGTC tATTTTAATC GCCAGATAAG CAATATATTG   1140

TGTAAATATT TGTATTCTTT ATCAGGAAAT TCAGGGAGAC GGGAAGTTAC TATCTACTAA   1200

AAGCCAAACA ATTTCTTACA GTTTTACTCT CTCTACTCTA GAGTAGCTTG GCACTGGCCG   1260

TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT CGCCTTGCAG   1320

CACATCCCCC TTTCGCCAGC TGGCGTAATA GCGAAGAGGC CCGCACCGAT CGCCCTTCCC   1380

AACAGTTGCG CAGCCTGAAT GGCGAATGGC GCCTGATGCG GTATTTTCTC CTTACGCATC   1440

TGTGCGGTAT TTCACACCGC ATATGGTGCA CTCTCAGTAC AATCTGCTCT GATGCCGCAT   1500

AGTTAAGCCA GCCCCGACAC CCGCCAACAC CCGCTGACGC GCCCTGACGG GCTTGTCTGC   1560

TCCCGGCATC CGCTTACAGA CAAGCTGTGA CCGTCTCCGG GAGCTGCATG TGTCAGAGGT   1620

TTTCACCGTC ATCACCGAAA CGCGCGAGAT GAAAGGGCCT CGTGATACGC CTATTTTTAT   1680

AGGTTAATGT CATGATAATA ATGGTTTCTT AGACGTCAGG TGGCACTTTT CGGGGAAATG   1740

TGCGCGGAAC CCCTATTTGT TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA   1800

GACAATAACC CTGATAAATG CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC   1860

ATTTCCGTGT CGCCCTTATT CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC   1920

CAGAAACGCT GGTGAAAGTA AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA   1980

TCGAACTGGA TCTCAACAGC GGTAAGATCC TTGAGAGTTT TCGCCCCGAA GAACGTTTTC   2040

CAATGATGAG CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG   2100

GGCAAGAGCA ACTCGGTCGC CGCATACACT ATTCTCAGAA TGACTTGGTT GAGTACTCAC   2160

CAGTCACAGA AAAGCATCTT ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA   2220
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TAACCATGAG TGATAACACT GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG   2280

AGCTAACCGC TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC   2340

CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACAC CACGATGCCT GTAGCAATGG   2400

CAACAACGTT GCGCAAACTA TTAACTGGCG AACTACTTAC TCTAGCTTCC CGGCAACAAT   2460

TAATAGACTG GATGGAGGCG GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG   2520

CTGGCTGGTT TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG   2580

CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG ACGGGGAGTC   2640

AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT AGGTGCCTCA CTGATTAAGC   2700

ATTGGTAACT GTCAGACCAA GTTTACTCAT ATATACTTTA GATTGATTTA AAACTTCATT   2760

TTTAATTTAA AAGGATCTAG GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT   2820

AACGTGAGTT TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT   2880

GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA CCGCTACCAG   2940

CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA   3000

GCAGAGCGCA GATACCAAAT ACTGTCCTTC TAGTGTAGCC GTAGTTAGGC CACCACTTCA   3060

AGAACTCTGT AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG   3120

CCAGTGGCGA TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG   3180

CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT   3240

ACACCGAACT GAGATACCTA CAGCGTGAGC ATTGAGAAAG CGCCACGCTT CCCGAAGGGA   3300

GAAAGGCGGA CAGGTATCCG GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC   3360

TTCCAGGGGG AAACGCCTGG TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG   3420

AGCGTCGATT TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCAACG   3480

CGGCCTTTTT ACGGTCCTGG CCTTTTGCTG GCCTTTTGCT CACATGTCTT TCCTGCGTTA   3540

TCCCCTGATT CTGTGGATAA CCGTATTACC GCCTTTGAGT GAGCTGATAC CGCTCGCCGC   3600

AGCCGAACCG ACCGAGCGCA GCGAGTCAGT GAGCGAGGAA GCGGAAGAGC GCCCAATACG   3660

CAAACCGCCT CTCCCCGCGC GTTGGCCGAT TCATTAATGC AGCTGGCACG ACAGGTTTCC   3720

CGACTGGAAA GCGGGCAGTG AGCGCAACGC AATTAATGTG AGTTAGCTCA CTCATTAGGC   3780

ACCCCAGGCT TTACACTTTA TGCTTCCGGC TCGTATGTTG TGTGGAATTG TGAGCGGATA   3840

ACAATTTCAC ACAGGAAACA GCTATGACAT GATTACCG   3878

(2) INFORMATION FOR SEQ ID NO:28:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 47 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GATCCTTATT AGATCTGCTT GGCGCCATCC TCAATTTGGG GGTTGAG   47

(2) INFORMATION FOR SEQ ID NO:29:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 47 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCGACTCAAC CCCCAAATTG AGGATGGCGC CAAGCAGATC TAATAAG   47

(2) INFORMATION FOR SEQ ID NO:30:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3883 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT   60

TGGTTCCGAT AAGAGACCCA GAACTCCGGC CCCCCACCGC CCACCGCCAC CCCCATACAT   120

ATGTGGTACG CAAGTAAGAG TGCCTGCGCA TGCCCCATGT GCCCCACCAA GAGcTTTGCA   180

TCCCATACAA GTCCCCAAAG TGGAGAACCG AACCAATTCT TCGCGGGCAG AACAAAAGCT   240

TCTGCACACG TCTCCACTCG AATTTGGAGC CGGCCGGCGT GTGCAAAAGA GGTGAATCGA   300

ACGAAAGACC CGTGTGTAAA GCCGCGTTTC CAAAATGTAT AAAACCGAGA GCATCTGGCC   360

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AATGTGCATC AGTTGTGGTC AGCAGCAAAA TCAAGTGAAT CATCTCAGTG CAACTAAAGG    420

GGAATTCGAG CTCGGTACCC GGGGATCCTT ATTAGATCTG CTTGGCGCCA TCCTCAATTT    480

GGGGGTTGAG TCGACCTGCA GGCATGCAAG CTATTCGATG CACACTCACA TTCTTCTCCT    540

AATACGATAA TAAAACTTTC CATGAAAAAT ATGGAAAAAT ATATGAAAAT TGAGAAATCC    600

AAAAAACTGA TAAACGCTCT ACTTAATTAA AATAGATAAA TGGGAGCGGC AGGAATGGCG    660

GAGCATGGCC AAGTTCCTCC GCCAATCAGT CGTAAAACAG AAGTCGTGGA AAGCGGATAG    720

AAAGAATGTT CGATTTGACG GGCAAGCATG TCTGCTATGT GGCGGATTGC GGAGGAATTG    780

CACTGGAGAC CAGCAAGGTT CTCATGACCA AGAATATAGC GGTGAGTGAG CGGGAAGCTC    840

GGTTTCTGTC CAGATCGAAC TCAAAACTAG TCCAGCCAGT CGCTGTCGAA ACTAATTAAG    900

TCAATGAGTT TTTCATGTTA GTTTCGCGCT GAGCAACAAT TAAGTTTATG TTTCAGTTCG    960

GCTTAGATTT CGCTGAAGGA CTTGCCACTT TCAATCAATA CTTTAGAACA AAATCAAAAC   1020

TCATTCTAAT AGCTTGGTGT TCATCTTTTT TTTTAATGAT AAGCATTTTG TCGTTTATAC   1080

TTTTTATATT TCGATATTAA ACCACCTATG AAGTCtATTT TAATCGCCAG ATAAGCAATA   1140

TATTGTGTAA ATATTTGTAT TCTTTATCAG GAAATTCAGG GAGACGGGAA GTTACTATCT   1200

ACTAAAGCC AAACAATTTC TTACAGTTTT ACTCTCTCTA CTCTAGAGTA GCTTGGCACT   1260

GGCCGTCGTT TTACAACGTC GTGACTGGGA AAACCCTGGC GTTACCCAAC TTAATCGCCT   1320

TGCAGCACAT CCCCCTTTCG CCAGCTGGCG TAATAGCGAA GAGGCCCGCA CCGATCGCCC   1380

TTCCCAACAG TTGCGCAGCC TGAATGGCGA ATGGCGCCTG ATGCGGTATT TTCTCCTTAC   1440

GCATCTGTGC GGTATTTCAC ACCGCATATG GTGCACTCTC AGTACAATCT GCTCTGATGC   1500

CGCATAGTTA AGCCAGCCCC GACACCCGCC AACACCCGCT GACGCGCCCT GACGGGCTTG   1560

TCTGCTCCCG GCATCCGCTT ACAGACAAGC TGTGACCGTC TCCGGGAGCT GCATGTGTCA   1620

GAGGTTTTCA CCGTCATCAC CGAAACGCGC GAGACGAAAG GGCCTCGTGA TACGCCTATT   1680

TTTATAGGTT AATGTCATGA TAATAATGGT TTCTTAGACG TCAGGTGGCA CTTTTCGGGG   1740

AAATGTGCGC GGAACCCCTA TTTGTTTATT TTTCTAAATA CATTCAAATA TGTATCCGCT   1800

CATGAGACAA TAACCCTGAT AAATGCTTCA ATAATATTGA AAAAGGAAGA GTATGAGTAT   1860

TCAACATTTC CGTGTCGCCC TTATTCCCTT TTTTGCGGCA TTTTGCCTTC CTGTTTTTGC   1920

TCACCCAGAA ACGCTGGTGA AAGTAAAAGA TGCTGAAGAT CAGTTGGGTG CACGAGTGGG   1980

TTACATCGAA CTGGATCTCA ACAGCGGTAA GATCCTTGAG AGTTTTCGCC CCGAAGAACG   2040

TTTTCCAATG ATGAGCACTT TTAAAGTTCT GCTATGTGGC GCGGTATTAT CCCGTATTGA   2100

CGCCGGGCAA GAGCAACTCG GTCGCCGCAT ACACTATTCT CAGAATGACT TGGTTGAGTA   2160

CTCACCAGTC ACAGAAAAGC ATCTTACGGA TGGCATGACA GTAAGAGAAT TATGCAGTGC   2220

TGCCATAACC ATGAGTGATA ACACTGCGGC CAACTTACTT CTGACAACGA TCGGAGGACC   2280
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GAAGGAGCTA ACCGCTTTTT TGCACAACAT GGGGGATCAT GTAACTCGCC TTGATCGTTG    2340

GGAACCGGAG CTGAATGAAG CCATACCAAA CGACGAGCGT GACACCACGA TGCCTGTAGC    2400

AATGGCAACA ACGTTGCGCA AACTATTAAC TGGCGAACTA CTTACTCTAG CTTCCCGGCA    2460

ACAATTAATA GACTGGATGG AGGCGGATAA AGTTGCAGGA CCACTTCTGC GCTCGGCCCT    2520

TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT GAGCGTGGGT CTCGCGGTAT    2580

CATTGCAGCA CTGGGGCCAG ATGGTAAGCC CTCCCGTATC GTAGTTATCT ACACGACGGG    2640

GAGTCAGGCA ACTATGGATG AACGAAATAG ACAGATCGCT GAGATAGGTG CCTCACTGAT    2700

TAAGCATTGG TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT    2760

TCATTTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA TGACCAAAAT    2820

CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA TCAAAGGATC    2880

TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA AACCACCGCT    2940

ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA AGGTAACTGG    3000

CTTCAGCAGA GCGCAGATAC CAAATACTGT CCTTCTAGTG TAGCCGTAGT TAGGCCACCA    3060

CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC    3120

TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT AGTTACCGGA    3180

TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA CAGCCCAGCT TGGAGCGAAC    3240

GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCATTGA GAAAGCGCCA CGCTTCCCGA    3300

AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG    3360

GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG    3420

ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA AAAACGCCAG    3480

CAACGCGGCC TTTTTACGGT CCTGGCCTTT TGCTGGCCTT TTGCTCACAT GTCTTTCCTG    3540

CGTTATCCCC TGATTCTGTG ATAACCGTA TTACCGCCTT TGAGTGAGCT GATACCGCTC     3600

GCCGCAGCCG AACCGACCGA GCGCAGCGAG TCAGTGAGCG AGGAAGCGGA AGAGCGCCCA    3660

ATACGCAAAC CGCCTCTCCC CGCGCGTTGG CCGATTCATT AATGCAGCTG GCACGACAGG    3720

TTTCCCGACT GGAAAGCGGG CAGTGAGCGC AACGCAATTA ATGTGAGTTA GCTCACTCAT    3780

TAGGCACCCC AGGCTTTACA CTTTATGCTT CCGGCTCGTA TGTTGTGTGG AATTGTGAGC    3840

GGATAACAAT TTCACACAGG AAACAGCTAT GACATGATTA CCG                      3883
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(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
GAATTCATGG GCCACACACG GAGGCAGGGA ACATCACCAT CCAAGTGTCC ATACCTCAAT      60
TTCTTTCAGC TCTTGGTGCT GGCTGGTCTT TCTCACTTCT GTTCAGGTGT TATCCACGTG     120
ACCAAGGAAG TGAAAGAAGT GGCAACGCTG TCCTGTGGTC ACAATGTTTC TGTTGAAGAG     180
CTGGCACAAA CTCGCATCTA CTGGCAAAAG GAGAAGAAAA TGGTGCTGAC TATGATGTCT     240
GGGGACATGA ATATATGGCC CGAGTACAAG AACCGGACCA TCTTTGATAT CACTAATAAC     300
CTCTCCATTG TGATCCTGGC TCTGCGCCCA TCTGACGAGG GCACATACGA GTGTGTTGTT     360
CTGAAGTATG AAAAAGACGC TTTCAAGCGG GAACACCTGG CTGAAGTGAC GTTATCAGTC     420
AAAGCTGACT TCCCTACACC TAGTATATCT GACTTTGAAA TTCCAACTTC TAATATTAGA     480
AGGATAATTT GCTCAACCTC TGGAGGTTTT CCAGAGCCTC ACCTCTCCTG GTTGGAAAAT     540
GGAGAAGAAT TAAATGCCAT CAACACAACA GTTTCCCAAG ATCCTGAAAC TGAGCTCTAT     600
GCTGTTAGCA GCAAACTGGA TTTCAATATG ACAACCAACC ACAGCTTCAT GTGTCTCATC     660
AAGTATGGAC ATTTAAGAGT GAATCAGACC TTCAACTGGA ATACAACCAA GCAAGAGCAT     720
TTTCCTGATA ACCTGCTCCC ATCCTGGGCC ATTACCTTAA TCTCAGTAAA TGGAATTTTT     780
GTGATATGCT GCCTGACCTA CTGCTTTGCC CCAAGATGCA GAGAGAGAAG GAGGAATGAG     840
AGATTGAGAA GGGAAAGTGT ACGCCCTGTA TAAGGATTC                           879
```

(2) INFORMATION FOR SEQ ID NO:32:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 738 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
GAATTCATGG GCCACACACG GAGGCAGGGA ACATCACCAT CCAAGTGTCC ATACCTCAAT      60
TTCTTTCAGC TCTTGGTGCT GGCTGGTCTT TCTCACTTCT GTTCAGGTGT TATCCACGTG     120
ACCAAGGAAG TGAAAGAAGT GGCAACGCTG TCCTGTGGTC ACAATGTTTC TGTTGAAGAG     180
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CTGGCACAAA CTCGCATCTA CTGGCAAAAG GAGAAGAAAA TGGTGCTGAC TATGATGTCT    240

GGGGACATGA ATATATGGCC CGAGTACAAG AACCGGACCA TCTTTGATAT CACTAATAAC    300

CTCTCCATTG TGATCCTGGC TCTGCGCCCA TCTGACGAGG GCACATACGA GTGTGTTGTT    360

CTGAAGTATG AAAAAGACGC TTTCAAGCGG GAACACCTGG CTGAAGTGAC GTTATCAGTC    420

AAAGCTGACT TCCCTACACC TAGTATATCT GACTTTGAAA TTCCAACTTC TAATATTAGA    480

AGGATAATTT GCTCAACCTC TGGAGGTTTT CCAGAGCCTC ACCTCTCCTG GTTGGAAAAT    540

GGAGAAGAAT TAAATGCCAT CAACACAACA GTTTCCCAAG ATCCTGAAAC TGAGCTCTAT    60{

GCTGTTAGCA GCAAACTGGA TTTCAATATG ACAACCAACC ACAGCTTCAT GTGTCTCATC    660

AAGTATGGAC ATTTAAGAGT GAATCAGACC TTCAACTGGA ATACAACCAA GCAAGAGCAT    720

TTTCCTGATA ACGGATTC                                                  738
```

(2) INFORMATION FOR SEQ ID NO:33:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1002 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

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GAGCTCATGG ATCCCCAGTG CACTATGGGA CTGAGTAACA TTCTCTTTGT GATGGCCTTC     60

CTGCTCTCTG GTGCTGCTCC TCTGAAGATT CAAGCTTATT TCAATGAGAC TGCAGACCTG    120

CCATGCCAAT TTGCAAACTC TCAAAACCAA AGCCTGAGTG AGCTAGTAGT ATTTTGGCAG    180

GACCAGGAAA ACTTGGTTCT GAATGAGGTA TACTTAGGCA AAGAGAAATT TGACAGTGTT    240

CATTCCAAGT ATATGGGCCG CACAAGTTTT GATTCGGACA GTTGGACCCT GAGACTTCAC    300

AATCTTCAGA TCAAGGACAA GGGCTTGTAT CAATGTATCA TCCATCACAA AAAGCCCACA    360

GGAATGATTC GCATCCACCA GATGAATTCT GAACTGTCAG TGCTTGCTAA CTTCAGTCAA    420

CCTGAAATAG TACCAATTTC TAATATAACA GAAATGTGT ACATAAATTT GACCTGCTCA      460

TCTATACACG GTTACCAGA ACCTAAGAAG ATGAGTGTTT TGCTAAGAAC CAAGAATTCA     540

ACTATCGAGT ATGATGGTAT TATGCAGAAA TCTCAAGATA ATGTCACAGA ACTGTACGAC    600

GTTTCCATCA GCTTGTCTGT TTCATTCCCT GATGTTACGA GCAATATGAC CATCTTCTGT    660

ATTCTGGAAA CTGACAAGAC GCGGCTTTTA TCTTCACCTT TCTCTATAGA GCTTGAGGAC    720

CCTCAGCCTC CCCCAGACCA CATTCCTTGG ATTACAGCTG TACTTCCAAC AGTTATTATA    780
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TGTGTGATGG TTTTCTGTCT AATTCTATGG AAATGGAAGA AGAAGAAGCG GCCTCGCAAC   840

TCTTATAAAT GTGGAACCAA CACAATGGAG AGGGAAGAGA GTGAACAGAC CAAGAAAAGA   900

GAAAAATCC ATATACCTGA AAGATCTGAT GAAGCCCAGC GTGTTTTTAA AAGTTCGAAG   960

ACATCTTCAT GCGACAAAAG TGATACATGT TTTTAAGGGC CC   1002

(2) INFORMATION FOR SEQ ID NO:34:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 751 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GAGCTCATGG ATCCCCAGTG CACTATGGGA CTGAGTAACA TTCTCTTTGT GATGGCCTTC   60

CTGCTCTCTG GTGCTGCTCC TCTGAAGATT CAAGCTTATT TCAATGAGAC TGCAGACCTG   120

CCATGCCAAT TTGCAAACTC TCAAAACCAA AGCCTGAGTG AGCTAGTAGT ATTTTGGCAG   180

GACCAGGAAA ACTTGGTTCT GAATGAGGTA TACTTAGGCA AAGAGAAATT TGACAGTGTT   240

CATTCCAAGT ATATGGGCCG CACAAGTTTT GATTCGGACA GTTGGACCCT GAGACTTCAC   300

AATCTTCAGA TCAAGGACAA GGGCTTGTAT CAATGTATCA TCCATCACAA AAAGCCCACA   360

GGAATGATTC GCATCCACCA GATGAATTCT GAACTGTCAG TGCTTGCTAA CTTCAGTCAA   420

CCTGAAATAG TACCAATTTC TAATATAACA GAAAATGTGT ACATAAATTT GACCTGCTCA   480

TCTATACACG GTTACCCAGA ACCTAAGAAG ATGAGTGTTT TGCTAAGAAC CAAGAATTCA   540

ACTATCGAGT ATGATGGTAT TATGCAGAAA TCTCAAGATA ATGTCACAGA ACTGTACGAC   600

GTTTCCATCA GCTTGTCTGT TTCATTCCCT GATGTTACGA GCAAATGAC CATCTTCTGT   660

ATTCTGGAAA CTGACAAGAC GCGGCTTTTA TCTTCACCTT TCTCTATAGA GCTTGAGGAC   720

CCTCAGCCTC CCCCAGACCA CATTGGGGCC C   751

(2) INFORMATION FOR SEQ ID NO:35:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1611 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

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GAATTCATGG CTCCCAGCAG CCCCCGGCCC GCGCTGCCCG CACTCCTGGT CCTGCTCGGG    60
GCTCTGTTCC CAGGACCTGG CAATGCCCAG ACATCTGTGT CCCCCTCAAA AGTCATCCTG   120
CCCCGGGGAG GCTCCGTGCT GGTGACATGC AGCACCTCCT GTGACCAGCC CAAGTTGTTG   180
GGCATAGAGA CCCCGTTGCC TAAAAAGGAG TTGCTCCTGC CTGGGAACAA CCGGAAGGTG   240
TATGAACTGA GCAATGTGCA AGAAGATAGC CAACCAATGT GCTATTCAAA CTGCCCTGAT   300
GGGCAGTCAA CAGCTAAAAC CTTCCTCACC GTGTACTGGA CTCCAGAACG GGTGGAACTG   360
GCACCCCTCC CCTCTTGGCA GCCAGTGGGC AAGAACCTTA CCCTACGCTG CCAGGTGGAG   420
GGTGGGGCAC CCCGGGCCAA CCTCACCGTG GTGCTGCTCC GTGGGGACAA GGAGCTGAAA   480
CGGGAGCCAG CTGTGGGGGA GCCCGCTGAG GTCACGACCA CGGTGCTGGA GAGGAGAGAT   540
CACCATGGAG CCAATTTCTC GTGCCGCACT GAACTGGACC TGCGGCCCCA AGGGCTGGAG   600
CTGTTTGAGA ACACCTCGGC CCCCTACCAG CTCCAGACCT TTGTCCTGCC AGCGACTCCC   660
CCACAACTTG TCAGCCCCCG GGTCCTAGAG GTGGACACGC AGGGGACCGT GGTCTGTTCC   720
CTGGACGGGC TGTTCCCAGT CTCGGAGGCC CAGGTCCACC TGGCACTGGG GGACCAGAGG   780
TTGAACCCCA CAGTCACCTA TGGCAACGAC TCCTTCTCGG CTAAGGCCTC AGTCAGTGTG   840
ACCGCAGAGG ACGAGGGCAC CCAGCGGCTG ACGTGTGCAG TAATACTGGG GAACCAGAGC   900
CAGGAGACAC TGCAGACAGT GACCATCTAC AGCTTTCCGG CGCCCAACGT GATTCTGACG   960
AAGCCAGAGG TCTCAGAAGG GACCGAGGTG ACAGTGAAGT GTGAGGCCCA CCCTAGAGCC  1020
AAGGTGACGC TGAATGGGGT TCCAGCCCAG CCACTGGGCC CGAGGGCCCA GCTCCTGCTG  1080
AAGGCCACCC CAGAGGACAA CGGGCGCAGC TTCTCCTGCT CTGCAACCCT GGAGGTGGCC  1140
GGCCAGCTTA TACACAAGAA CCAGACCCCG GAGCTTCGTG TCCTGTATGG CCCCCGACTG  1200
GACGAGAGGG ATTGTCCGGG AAACTGGACG TGGCCAGAAA ATTCCCAGCA GACTCCAATG  1260
TGCCAGGCTT GGGGGAACCC ATTGCCCGAG CTCAAGTGTC TAAAGGATGG CACTTTCCCA  1320
CTGCCCATCG GGAATCAGT GACTGTCACT CGAGATCTTG AGGGCACCTA CCTCTGTCGG  1380
GCCAGGAGCA CTCAAGGGGA GGTCACCCGC GAGGTGACCG TGAATGTGCT CTCCCCCGG  1440
TATGAGATTG TCATCATCAC TGTGGTAGCA GCCGCAGTCA TAATGGGCAC TGCAGGCCTC  1500
AGCACGTACC TCTATAACCG CCAGCGGAAG ATCAAGAAAT ACAGACTACA ACAGGCCCAA  1560
AAAGGGACCC CCATGAAACC GAACACACAA GCCACGCCTC CCTGAGGATC C            1611
```

(2) INFORMATION FOR SEQ ID NO:36:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1452 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

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GAATTCATGG CTCCCAGCAG CCCCCGGCCC GCGCTGCCCG CACTCCTGGT CCTGCTCGGG    60
GCTCTGTTCC CAGGACCTGG CAATGCCCAG ACATCTGTGT CCCCCTCAAA AGTCATCCTG   120
CCCCGGGGAG GCTCCGTGCT GGTGACATGC AGCACCTCCT GTGACCAGCC CAAGTTGTTG   180
GGCATAGAGA CCCCGTTGCC TAAAAGGAG TTGCTCCTGC CTGGGAACAA CCGGAAGGTG    240
TATGAACTGA GCAATGTGCA AGAAGATAGC CAACCAATGT GCTATTCAAA CTGCCCTGAT   300
GGGCAGTCAA CAGCTAAAAC CTTCTTCACC GTGTACTGGA CTCCAGAACG GGTGGAACTG   360
GCACCCCTCC CCTCTTGGCA GCCAGTGGGC AAGAACCTTA CCCTACGCTG CCAGGTGGAG   420
GGTGGGGCAC CCCGGGCCAA CTTCATCCTG GTGCTGCTCC GTGGGGAGAA GGAGCTGAAA   480
CGGGAGCCAG CTGTGGGGGA GCCCCTTCAG GTCACGACCA CGGTGCTGGT GAGGAGAGAT   540
CACCATGGAG CCAATTTCTC GTGCCGCACT GAACTGGACC TGCGGCCCCA AGGGCTGGAG   600
CTGTTTGAGA ACACCTCGGC CCCTTATCAG CTCCAGACCT TTGTCCTGCC AGCGACTCCC   660
CCACAACTTG TCAGCCCCCG GGTCCTATAG GTGGACACGC AGGGGACCGT GGTCTGTTCC   720
CTGGACGGGC TGTTCCCAGT CTTCCAGCCC CAGGTCCACC TGGCACTGGG GGACCAGAGG   780
TTGAACCCCA CAGTCACCTA TGTCAATGAT TCCTTCTCGG CCAAGGCCTC AGTCAGTGTG   840
ACCGCAGAGG ACGAGGGCAC CTACTTCTTC ACGTGTGCAG TAATACTGGG GAACCAGAGC   900
CAGGAGACAC TGCAGACACT CATTATTTAT AGCTTTCCGG CGCCCAACGT GATTCTGACG   960
AAGCCAGAGG TCTCAGAAGG CATTCAGCTG ACAGTGAAGT GTGAGGCCCA CCCTAGAGCC  1020
AAGGTGACGC TGAATGGGGT TCTACTTCAG CCACTGGGCC CGAGGGCCCA GCTCCTGCTG  1080
AAGGCCACCC CAGAGGACAA CGGGCGCAGC TTCTCCTGCT CTGCAACCCT GGAGGTGGCC  1140
GGCCAGCTTA TACACAAGAA CCAGACCCGG GAGCTTCGTG TCCTGTATGG CCCCCGACTG  1200
GACGAGAGGG ATTGTCCGGG AAATGGACC TGGCCAGAAA ATTCCCAGCA GACTCCAATG  1260
TGCCAGGCTT GGGGGAACCC ATTGCCCCAG CTCAAGTGTC TAAAGGATGG CACTTTCCCA  1320
CTGCCCATCG GGGAATCAGT GACTGTCACT CGAGATCTTG AGGGCACCTA CCTCTGTCGG  1380
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GCCAGGAGCA CTCAAGGGGA GGTCACCCGC GAGGTGACCG TGAATGTGCT CTCCCCCCGG   1440

TATGAGGGAT CC   1452

(2) INFORMATION FOR SEQ ID NO:37:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 726 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: double
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GAGCTCATGG TTGCTGGGAG CGACGCGGGG CGGGCCCTGG GGGTCCTCAG CGTGGTCTGC   60

CTGCTGCACT GCTTTGGTTT CATCAGCTGT TTTTCCCAAC AAATATATGG TGTTGTGTAT   120

GGGAATGTAA CTTTCCATGT ACCAAGCAAT GTGCCTTTAA AAGAGGTCCT ATGGAAAAAA   180

CAAAAGGATA AAGTTGCAGA ACTGGAAAAT TCTGAATTCA GAGCTTTCTC ATCTTTTAAA   240

AATAGGGTTT ATTTAGACAC TGTGTCAGGT AGCCTCACTA TCTACAACTT AACATCATCA   300

GATGAAGATG AGTATGAAAT GGAATCGCCA AATATTACTG ATACCATGAA GTTCTTTCTT   360

TATGTGCTTG AGTCTCTTCC ATCTCCCACA CTAACTTGTG CATTGACTAA TGGAAGCATT   420

GAAGTCCAAT GCATGATACC AGAGCATTAC AACAGCCATC GAGGACTTAT AATGTACTCA   480

TGGGATTGTC CTATGGAGCA ATGTAAACGT AACTCAACCA GTATATATTT TAAGATGGAA   540

AATGATCTTC CACAAAAAAT ACAGTGTACT CTTAGCAATC CATTATTTAA TACAACATCA   600

TCAATCATTT TGACAACCTG TATCCCAAGC AGCGGTCATT CAAGACACAG ATATGCACTT   660

ATACCCATAC CATTAGCAGT AATTACAACA TGTATTGTGC TGTATATGAA TGTTCTTTAA   720

GGATCC   726

(2) INFORMATION FOR SEQ ID NO:38:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 657 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: double
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

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GAGCTCATGG TTGCTGGGAG CGACGCGGGG CGGGCCCTGG GGGTCCTCAG CGTGGTCTGC    60

CTGCTGCACT GCTTTGGTTT CATCAGCTGT TTTTCCCAAC AAATATATGG TGTTGTGTAT   120

GGGAATGTAA CTTTCCATGT ACCAAGCAAT GTGCCTTTAA AAGAGGTCCT ATGGAAAAAA   180

CAAAAGGATA AAGTTGCAGA ACTGGAAAAT TCTGAATTCA GAGCTTTCTC ATCTTTTAAA   240

AATAGGGTTT ATTTAGACAC TGTGTCAGGT AGCCTCACTA TCTACAACTT AACATCATCA   300

GATGAAGATG AGTATGAAAT GGAATCGCCA AATATTACTG ATACCATGAA GTTCTTTCTT   360

TATGTGCTTG AGTCTCTTCC ATCTCCCACA CTAACTTGTG CATTGACTAA TGGAAGCATT   420

GAAGTCCAAT GCATGATACC AGAGCATTAC AACAGCCATC GAGGACTTAT AATGTACTCA   480

TGGGATTGTC CTATGGAGCA ATGTAAACGT AACTCAACCA GTATATATTT TAAGATGGAA   540

AATGATCTTC CACAAAAAAT ACAGTGTACT CTTAGCAATC CATTATTTAA TACAACATCA   600

TCAATCATTT TGACAACCTG TATCCCAAGC AGCGGTCATT CAAGACACAG AGGATCC     657
```

(2) INFORMATION FOR SEQ ID NO:39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

    Glu Gln Leu Glu Ser Ile Ile Asn Phe Glu Lys Leu Thr Glu Trp Thr
    1   5   10   15

    Ser Ser Asn Val Met Glu Glu Arg
                20

(2) INFORMATION FOR SEQ ID NO:40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Asp Leu Arg Gly Tyr Val Tyr Gln Gly Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Phe Arg Ile Gly Cys Arg His Ser Arg
1   5

(2) INFORMATION FOR SEQ ID NO 42:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Ile Leu Lys Glu Pro Val His Gly Val
1   5

(2) INFORMATION FOR SEQ ID NO 43:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ATATGGATCC TCACCATCTC AGGGTGAGGG GC
32

(2) INFORMATION FOR SEQ ID NO:44:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 10 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

  (iii) HYPOTHETICAL:, NO

   (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

   Arg Gly Tyr Val Tyr Gln Gly Leu Lys Ser
   1   5   10

(2) INFORMATION FOR SEQ ID NO:45:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 9 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

  (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

   Phe Ala Pro Gly Asn Tyr Pro Ala Leu
   1   5

(2) INFORMATION FOR SEQ ID NO:46:

    (i) SEQUENCE CHARACTERISTICS:

                    (A) LENGTH: 8 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

       (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

         (v) FRAGMENT TYPE: internal

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

        Leu Ser Pro Phe Pro Phe Asp Leu
        1   5

(2) INFORMATION FOR SEQ ID NO:47:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 9 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: peptide

      (iii) HYPOTHETICAL: NO

       (iv) ANTI-SENSE: NO

        (v) FRAGMENT TYPE: internal

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

        Gln Leu Ser Pro Phe Pro Phe Asp Leu
        1   5

(2) INFORMATION FOR SEQ ID NO:48:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Ile Leu Lys Glu Pro Val His Gly Val
1   5

(2) INFORMATION FOR SEQ ID NO:49:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Tyr Met Asn Gly Thr Met Ser Gln Val
1   5

(2) INFORMATION FOR SEQ ID NO:50:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

```
Gly Ile Leu Gly Phe Val Phe Thr Leu
1               5
```

(2) INFORMATION FOR SEQ ID NO:51:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

```
Phe Leu Pro Ser Asp Phe Phe Pro Ser Val
1               5                   10
```

WE CLAIM:

1. A synthetic antigen presenting cell line for use with T-cell lymphocytes comprising:

a) a Class I MHC heavy chain gene operably linked to a first promoter and capable of expressing a Class I MHC heavy chain;

b) a β-2 microglobulin gene operably linked to a second promoter and capable of expressing a β-2 microglobulin that with the MHC heavy chain forms MHC molecules; and

c) a gene for an assisting molecule operably linked to a third promoter and capable of expressing an assisting molecule that interacts with a molecule on the T-cell lymphocytes;

at least one of the MHC gene, β-2 microglobulin gene and assisting molecule genes not being present in the cells from which the cell line is derived, such that the MHC molecules bind to a peptide, and the MHC molecules and assisting molecules are presented on the surface of the cell in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the MHC molecules.

2. The cell line of claim 1 wherein the assisting molecule is a costimulatory molecule.

3. The cell line of claim 2 wherein the costimulatory molecule is B7.1 or B7.2.

4. The cell line of claim 1 wherein the cell line is derived from a first species and the MHC heavy chain gene is from a second species.

5. The cell line of claim 4 wherein the first species is a poikilotherm and the second species is a homeotherm.

6. The cell line of claim 1 wherein the assisting molecule is an adhesion molecule.

7. The cell line of claim 6 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

8. The cell line of claim 1 having a gene for a first assisting molecule and a gene for a second assisting molecule.

9. The cell line of claim 8 wherein the first assisting molecule is a costimulatory molecule and the second assisting molecule is an adhesion molecule.

10. The cell line of claim 1 wherein at least one promoter is inducible.

11. The cell line of claim 10 wherein the first promoter is inducible.

12. The cell line of claim 1 wherein the peptide is bound to the MHC molecules within the cell.

13. The cell line of claim 1 wherein the MHC molecules are presented empty on the surface of the cell.

14. A stable poikilotherm cell line used to stimulate human T-cell lymphocytes comprising:

a) a Class I MHC gene operably linked to a first inducible promoter and capable of expressing a Class I MHC heavy chain;

b) a β-2 microglobulin gene operably linked to a second promoter and capable of expressing a β-2 microglobulin that forms MHC molecules with the MHC heavy chain; and

c) a gene for an assisting molecule operably linked to a third promoter and capable of expressing an assisting molecule that interacts with a molecule on the T-cell lymphocytes;

such that the cell assembles empty MHC molecules that are capable of binding to a peptide, and the MHC molecules and assisting molecules are presented on the surface of the cells in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the MHC molecules.

15. The cell line of claim 14 wherein the assisting molecule is a costimulatory molecule.

16. The cell line of claim 15 wherein the costimulatory molecule is B7.1 or B7.2.

17. The cell line of claim 14 wherein the assisting molecule is an adhesion molecule.

18. The cell line of claim 17 wherein the adhesion molecule is ICAM-1.

19. The cell line of claim 14 having a gene for a first assisting molecule and a gene for a second assisting molecule.

20. The cell line of claim 19 wherein the first assisting molecule is a costimulatory molecule and the second assisting molecule is an adhesion molecule.

21. A stable poikilotherm cell line used to stimulate human T-cell lymphocytes comprising:

a) a Class I MHC gene operably linked to a first promoter and capable of expressing a Class I MHC heavy chain;

b) a β-2 microglobulin gene operably linked to a second promoter and capable of expressing a β-2 microglobulin that forms MHC molecules with the MHC heavy chain; and

c) a gene for a costimulatory molecule operably linked to a third promoter and capable of expressing a costimulatory molecule that interacts with a molecule on the T-cell lymphocytes;

d) a gene for an adhesion molecule operably linked to a fourth promoter and capable of expressing an adhesion molecule that interacts with a cooperative adhesion molecule on the T-cell lymphocytes;

such that the cell is capable of assembling the MHC heavy chain and the β-2 microglobulin into MHC molecules that bind to a peptide, and transporting MHC molecules, costimulatory molecules and adhesion molecules

to the surface of the cell in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the MHC molecules.

22. The cell line of claim 21 wherein the costimulatory molecule is B7.1 or B7.2.

23. The cell line of claim 21 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

24. The cell line of claim 21 wherein at least one of the promoters is inducible.

25. The cell line of claim 21 wherein the peptide is bound to the MHC molecules within the cell.

26. The cell line of claim 21 wherein the MHC molecules are presented empty on the surface of the cell.

27. Fragments of cells of claim 1 having MHC molecules and assisting molecules operably associated such that they activate the population of T-cell lymphocytes.

28. The cell fragment of claim 27 wherein the MHC molecules are empty.

29. The cell fragment of claim 27 wherein the peptide is bound to the MHC molecules.

30. Fragments of cells of claim 21 having MHC molecules, costimulatory molecules and adhesion molecules operably associated such that they activate the population of T-cell lymphocytes.

31. The cell fragment of claim 27 wherein the peptide is bound to the MHC molecules.

32. A method of producing a synthetic antigen presenting a cell line comprising:

a) establishing of a culture of cells;

b) transfecting the culture with an expressible Class I MHC heavy chain gene operably linked to a first promoter;

c) transfecting the culture with an expressible β-2 microglobulin gene operably linked to a second promoter; and

d) transfecting the culture with an
5 expressible assisting molecule gene operably linked to a third promoter.

33. The method of claim 32 wherein the assisting molecule is a costimulatory molecule.

34. The method of claim 33 wherein the
10 costimulatory molecule is B7.1 or B7.2.

35. The method of claim 32 wherein the cell line is derived from a first species and the MHC heavy chain gene is from a second species.

36. The method of claim 35 wherein the first
15 species is a poikilotherm and the second species is a homeotherm.

37. The method of claim 32 wherein the assisting molecule is an adhesion molecule.

38. The method of claim 37 wherein the
20 adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

39. The method of claim 32 including the step of transfecting the culture with a gene for a second assisting molecule

40. The method of claim 39 wherein the first
25 assisting molecule is a costimulatory molecule and the second assisting molecule is an adhesion molecule.

41. The method of claim 32 wherein at least one of the promoters is inducible.

42. A method of producing a synthetic antigen
30 presenting cell line comprising:

a) establishing a culture of cells lacking a gene to at least one of: Class I MHC heavy chain, β-2 microglobulin and assisting molecule; and

b) transfecting the culture with an
35 expressible gene for each of the genes of (a) lacking in

the culture of cells, the gene being operably linked to a promoter.

43. A method of producing a synthetic antigen presenting a cell line comprising:

a) establishing of a culture of cells;

b) transfecting the culture with an expressible Class I MHC heavy chain gene operably linked to a promoter;

c) transfecting the culture with an expressible β-2 microglobulin gene operably linked to a second promoter;

d) transfecting the culture with an expressible costimulatory molecule gene operably linked to a third promoter; and

e) transfecting the culture with an expressible adhesion molecule gene operably linked to a third promoter.

44. A method of producing a synthetic antigen presenting cell line comprising:

a) establishing a culture of cells lacking a gene to at least one of: Class I MHC heavy chain, β-2 microglobulin and co-stimulatory molecule;

b) transfecting the culture with an expressible gene for each of the genes of (a) lacking in the culture of cells, the gene being linked to a first operable promoter; and

c) transfecting the culture with an expressible adhesion molecule gene, the gene being linked to a second operable promoter.

45. A method for activating CD8 · T-cells against a selected peptide in vitro, the method comprising:

a) providing the cell line of claim 1;

b)   culturing the cell line under conditions such that MHC molecules are produced on the surface of the cell line that are bound to the selected peptide; and

c)   contacting the cultured cells with the
5 CD8˙ T-cells against the selected peptide.

46.   The method of claim 45 wherein the cell line is a poikilotherm.

47.   The method of claim 45 further comprising the step of separating the activated CD8˙ T-cells from
10 the cell line.

48.   The method of claim 47 further comprising the step of adding the activated CD8˙ T-cells to an acceptable carrier or excipient to form a suspension.

49.   The method of claim 48 further comprising
15 the step of administering the suspension to a patient.

50.   A synthetic antigen-presenting matrix comprising:

a)   a support;

b)   extracellular portion of MHC molecules
20 capable of binding to a selected peptide and being operably linked to the support; and

c)   an assisting molecule operably linked to the support such that the extracellular·portion of the MHC and assisting molecules are present in sufficient
25 numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the extracellular portion of the MHC molecule.

51.   The matrix of claim 50 wherein the support is a cell fragment.

30 52.   The matrix of claim 50 wherein the support is a cell.

53.   The matrix of claim 52 wherein the extracellular portion of the MHC molecule is linked to the cell by a transmembrane domain of an MHC molecule.

53. The matrix of claim 53 wherein the extracellular portion of the MHC molecule is part of a complete MHC molecule.

54. The matrix of claim 50 wherein the support is liposome.

55. The matrix of claim 50 wherein the support is a solid surface.

56. The matrix of claim 50 wherein the extracellular portion of the MHC molecule is linked to an epitope which reacts with an antibody to link the portion to the support.

57. The matrix of claim 50 wherein the extracellular portion of the MHC molecule is linked to (His)$_6$ which reacts with nickel to link the portion to the support.

58. The matrix of claim 50 wherein the support is a porous material.

59. The matrix of claim 50 wherein the assisting molecule is a costimulatory molecule.

60. The matrix of claim 59 wherein the costimulatory molecule is B7.1 or B7.2.

61. The matrix of claim 59 wherein the costimulatory molecule is is at least a portion of an anti-CD80 antibody

62. The matrix of claim 50 wherein the assisting molecule is an adhesion molecule.

63. The matrix of claim 62 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

64. The matrix of claim 50 having two assisting molecules, the first assisting molecule being a costimulatory molecule and the second assisting molecule being an adhesion molecule.

65. The matrix of claim 50 wherein the extracellular portion of the MHC molecule is empty.

66. The matrix of claim 50 wherein the peptide is bound to the extracellular portion of the MHC molecule.

67. The matrix of claim 64 wherein the peptide is bound to the extracellular portion of the MHC molecule.

68. A synthetic antigen-presenting matrix comprising:

    a)   a support;

    b)   extracellular portion of MHC molecules capable of binding to a selected peptide and being operably linked to the support;

    c)   B-7.1 or B-7.2 molecules or a combination thereof operably linked to the support; and

    d)   ICAM-1 molecules operably linked to the support such that the molecules are present in sufficient amount to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the extracellular portion of the MHC molecule.

69. A method of producing a synthetic T-cell lymphocyte antigen activating matrix comprising:

    a)   establishing a first culture of cells;

    b)   transfecting the culture with an expressible Class I MHC heavy chain gene for at least the extracellular portion of the heavy chain operably linked to a promoter;

    c)   transfecting the culture with an expressible β-2 microglobulin gene operably linked to a second promoter;

    d)   harvesting the produced MHC molecule portions;

    e)   establishing a second culture of cells;

    f)   transfecting the second culture with an expressible assisting molecule gene for at least the

MISSING   PAGE

    b) contacting the matrix cells with the CD8⁺
T-cells.

    79. A method for generating cytotoxic CD8⁺
T-cells against a selected peptide, the method
comprising:

    a) providing the matrix of claim 67;

    b) contacting naive CD8⁺ T-cells with the
matrix *in vitro*.

    80. The matrix of claim 79 wherein the support
is a cell.

    81. The method of claim 79 wherein the
costimulatory molecule is B7.1 or B7.2.

    82. The method of claim 79 wherein the
costimulatory molecule is at least a portion of an anti-
CD28 antibody.

    83. The method of claim 79 wherein the
adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

    84. A method of treating a tumor in a patient
comprising:

    a) identifying a tumor specific antigen;

    b) collecting CD8⁺ T-cells from the patient;

    c) contacting the CD8⁺ T-cells with the
matrix of claim 67 *in vitro* in a sufficient amount and
for a sufficient time to generate cytotoxic CD8⁺ T-cells;
and

    d) returning the cytotoxic CD8⁺ T-cells to
the patient.

    85. The method of claim 84 wherein the antigen
is a self antigen of the patient.

    86. The method of claim 85 wherein the
assisting molecule is B7.1 or B7.2 and the adhesion
molecule is ICAM-1, ICAM-2 or ICAM-3.

    87. The method of claim 84 wherein the
assisting molecule is B7.1 and the adhesion molecule is
ICAM-1.
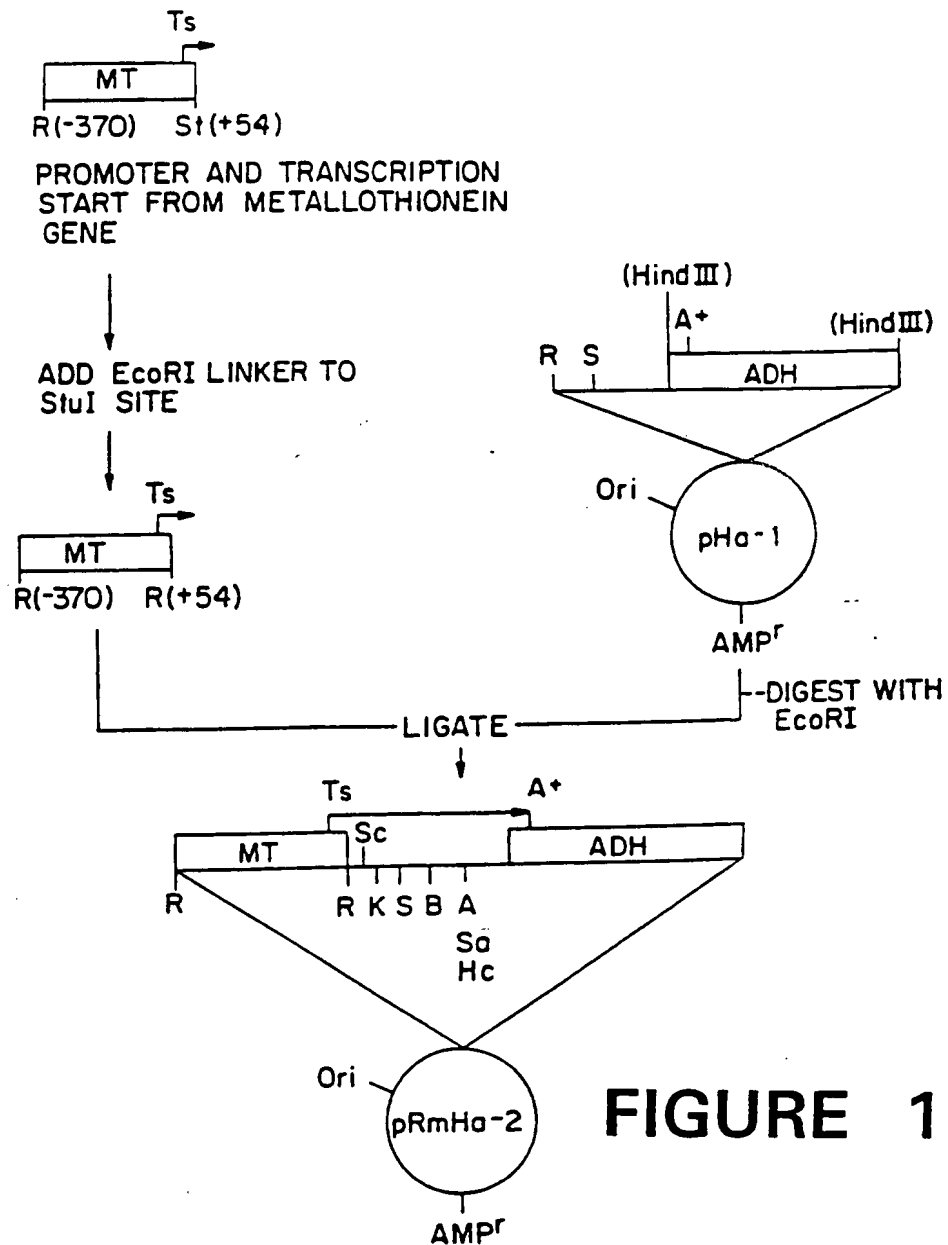
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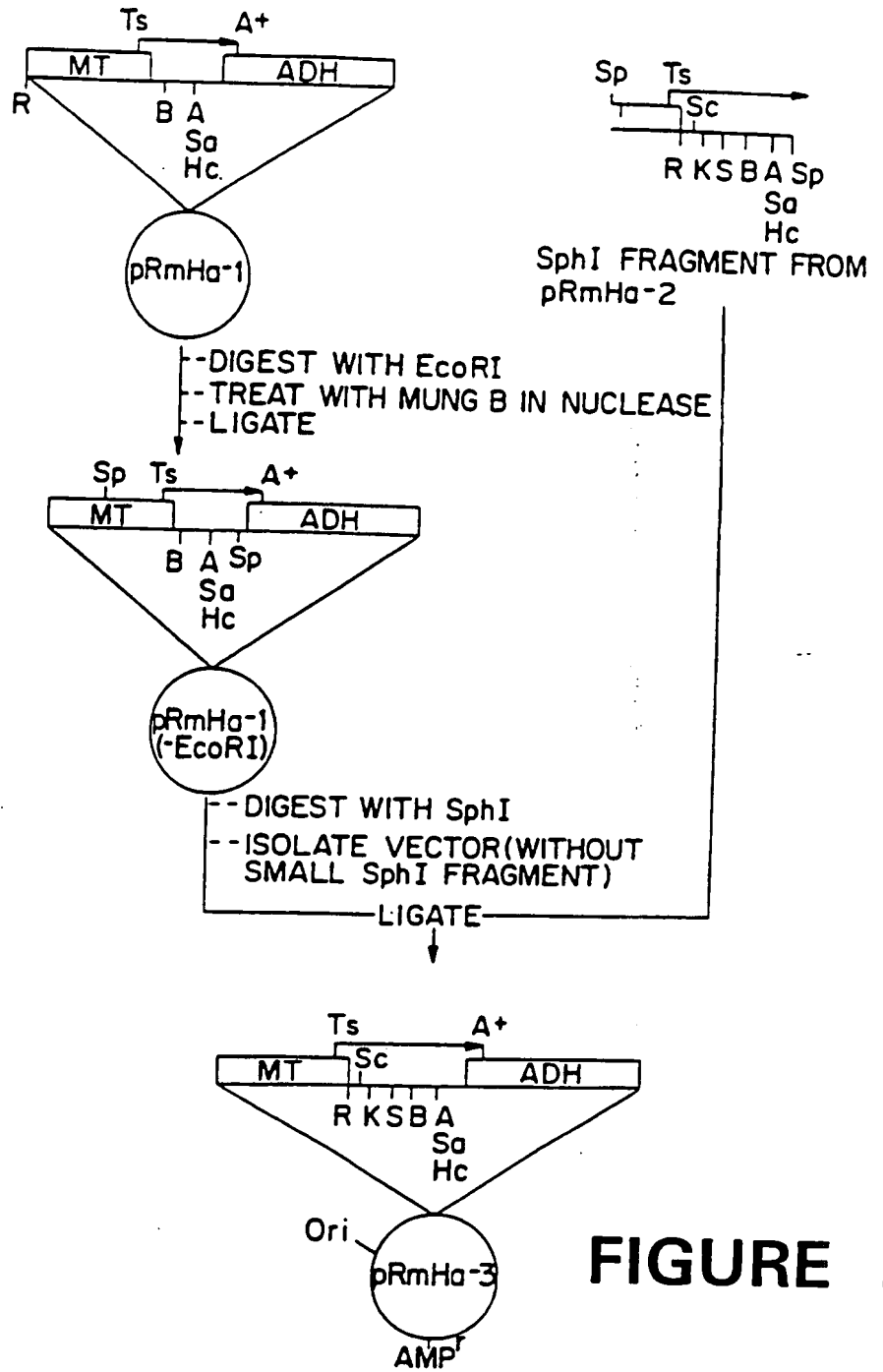
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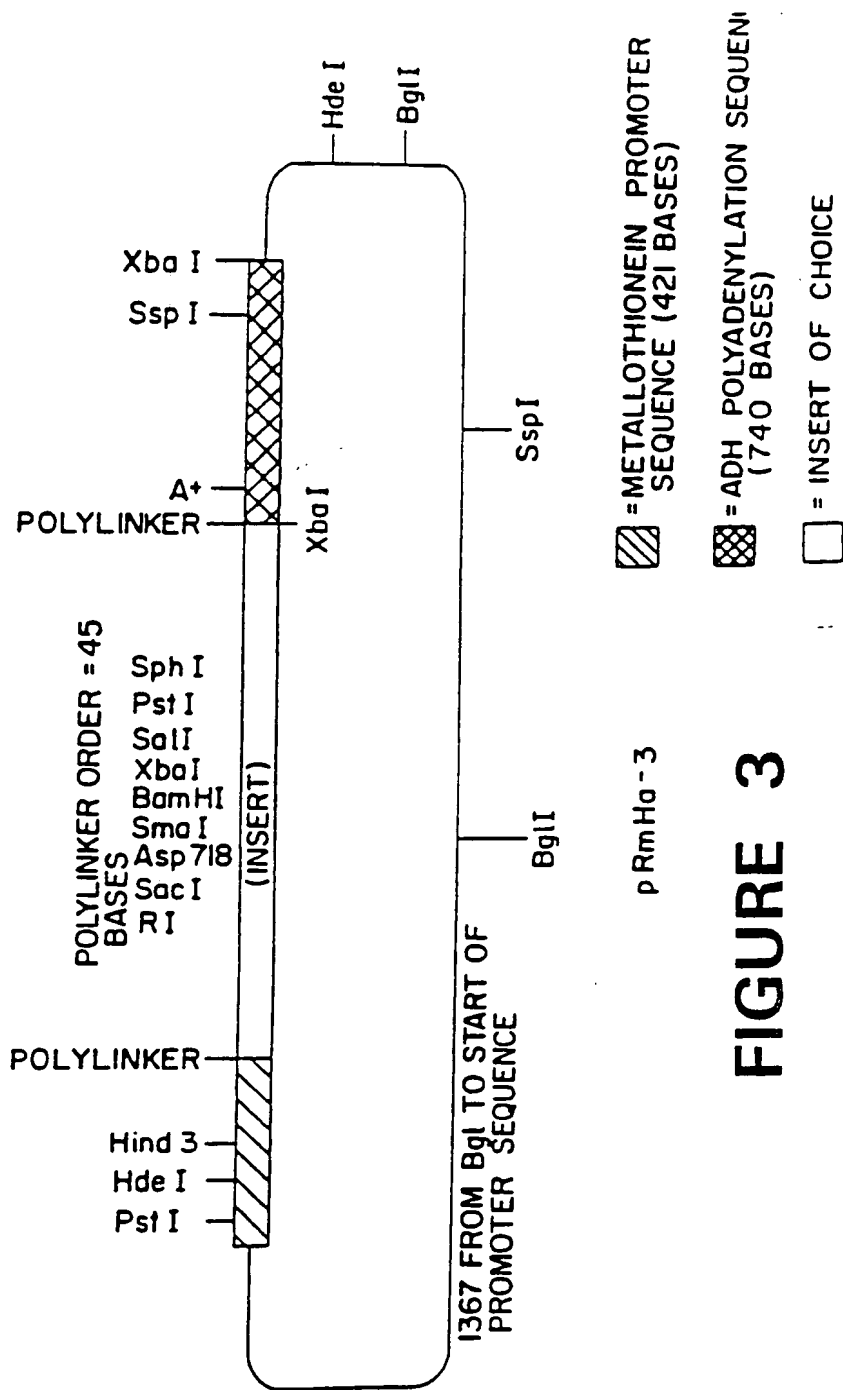
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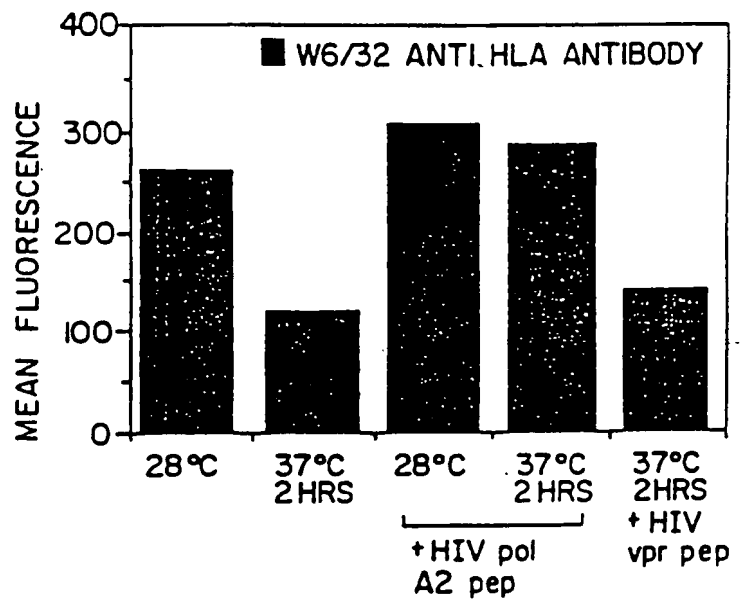
FIGURE 4

FIGURE 5

FIGURE 6

Fly Cell Transfectants
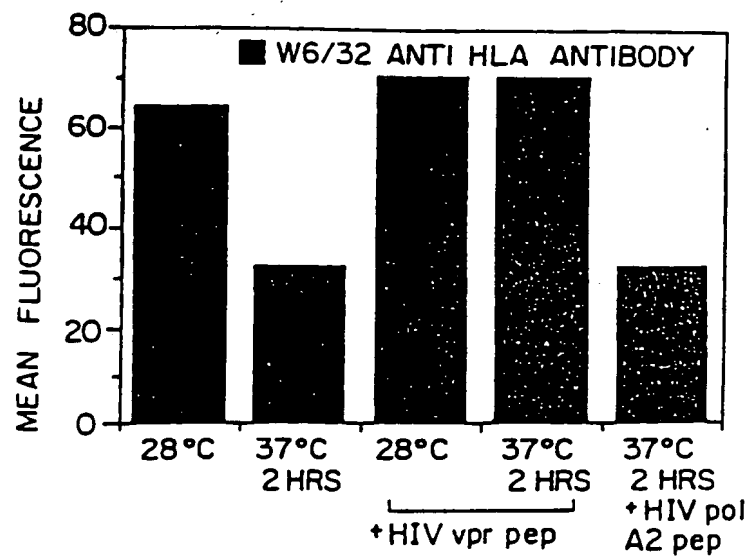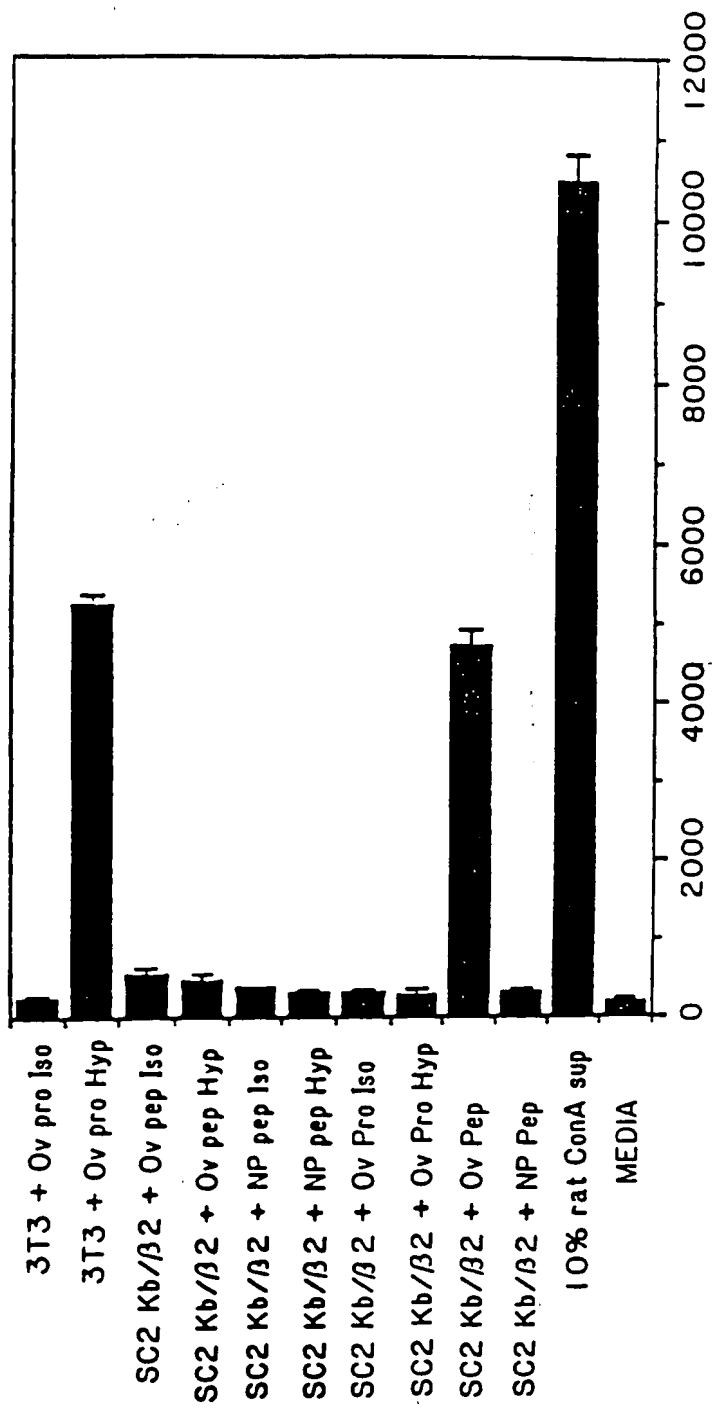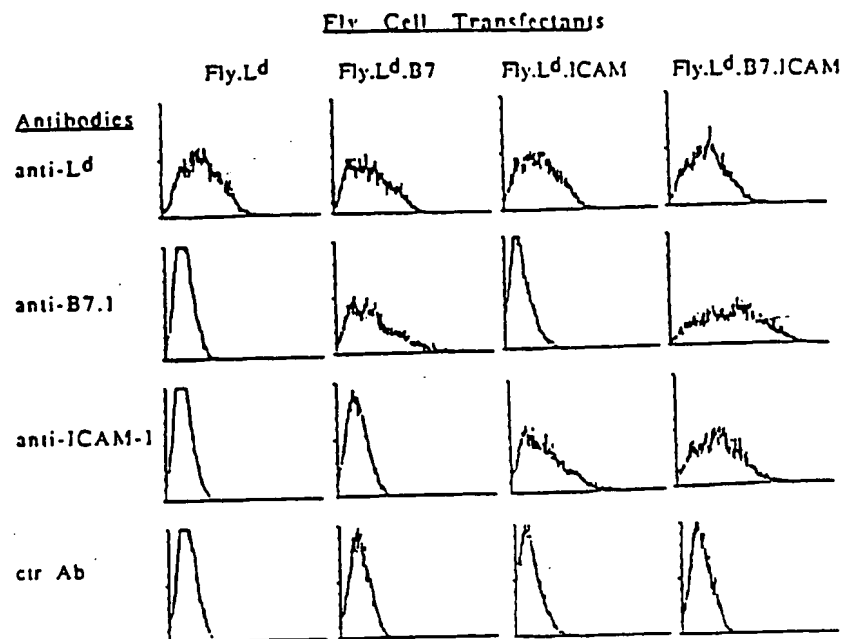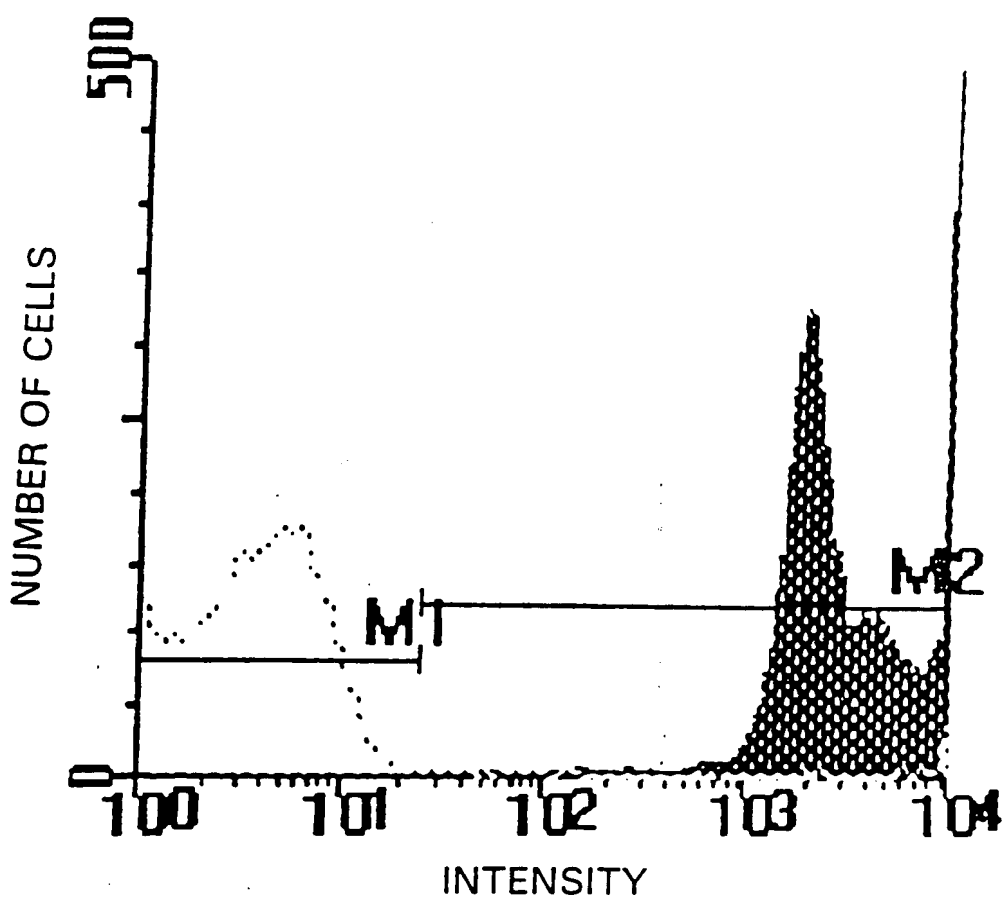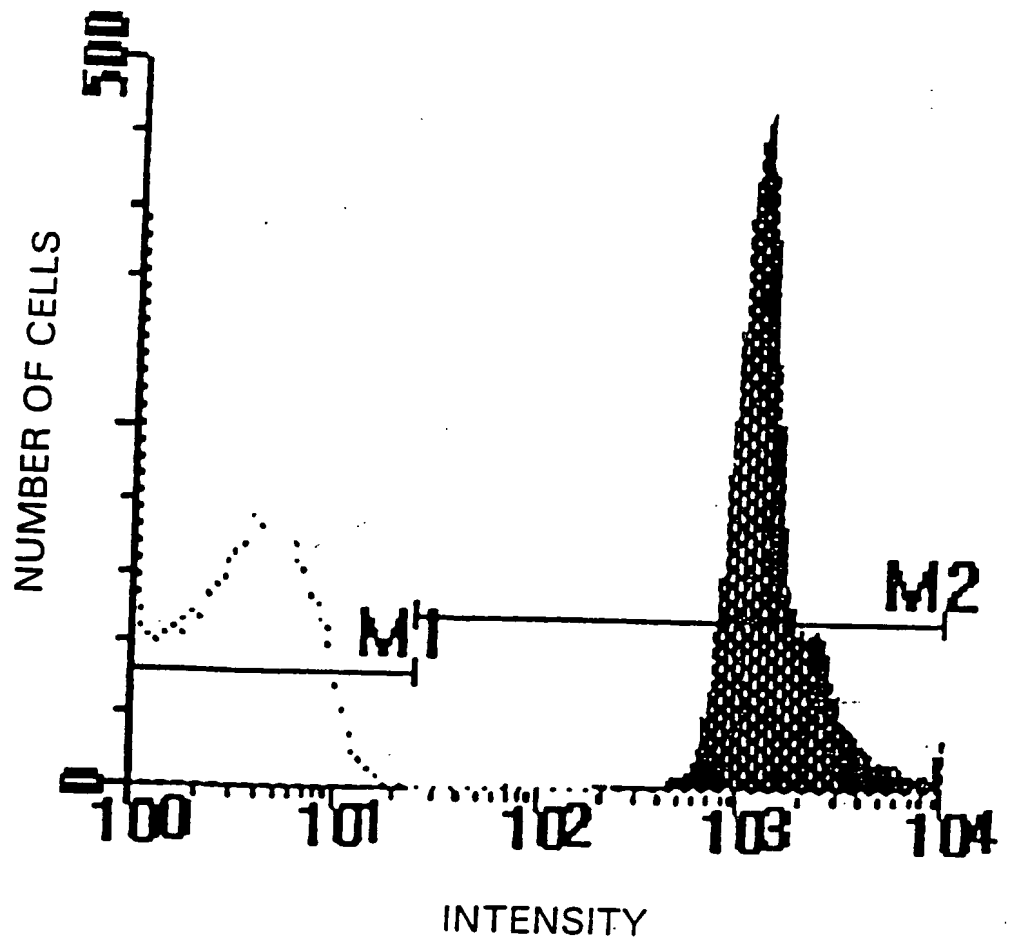
FIGURE 7

FIGURE 8

FIGURE 9

Kb Direct Binding to Microwell Plates

mAb1: Y3 (anti H2Kb ATCC HB176)
mAb2: 5F1 (Sherman & Randolph 1981)
mAb3: EH144 (Ajitkumar et al. 1989)

FIGURE 10

FIGURE 11

[3H] TdR Incorporation (cpm)
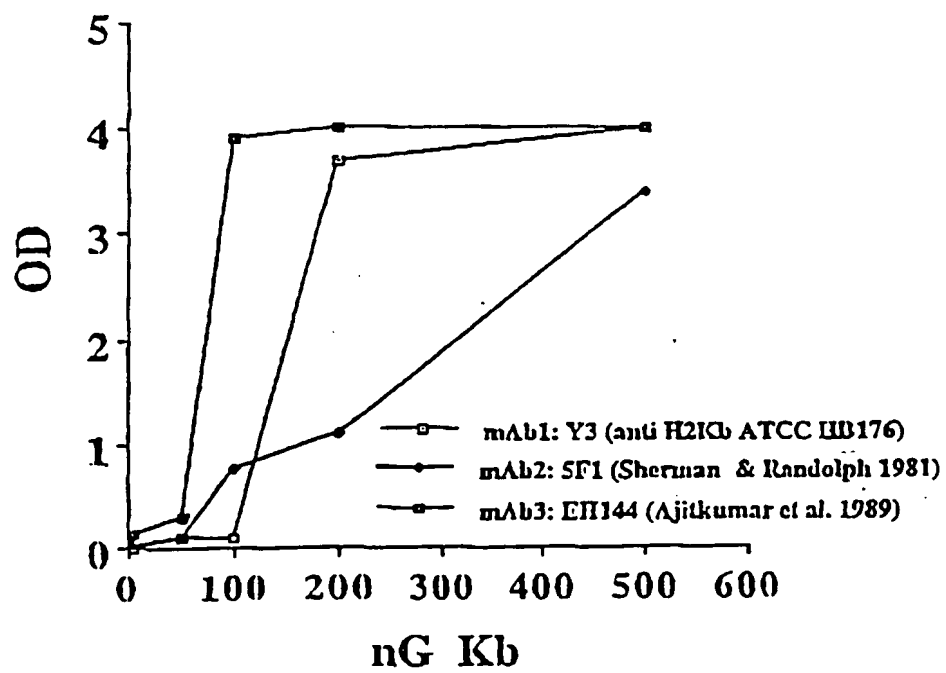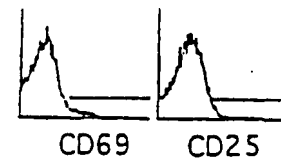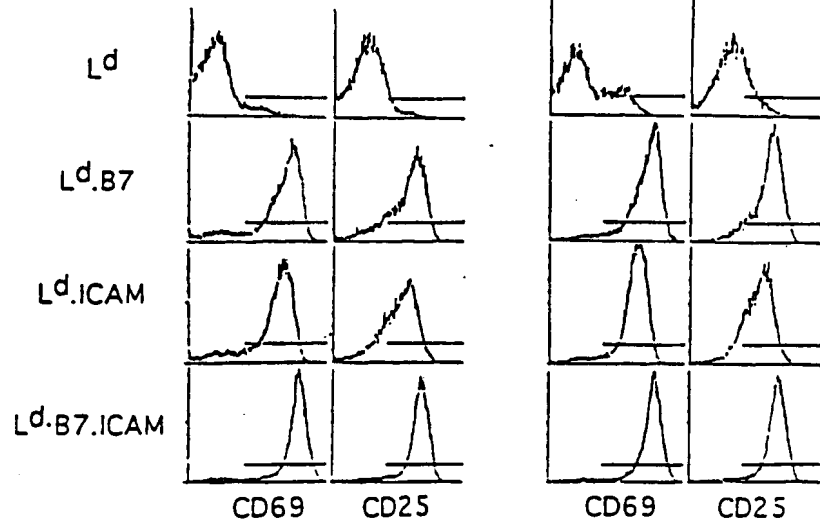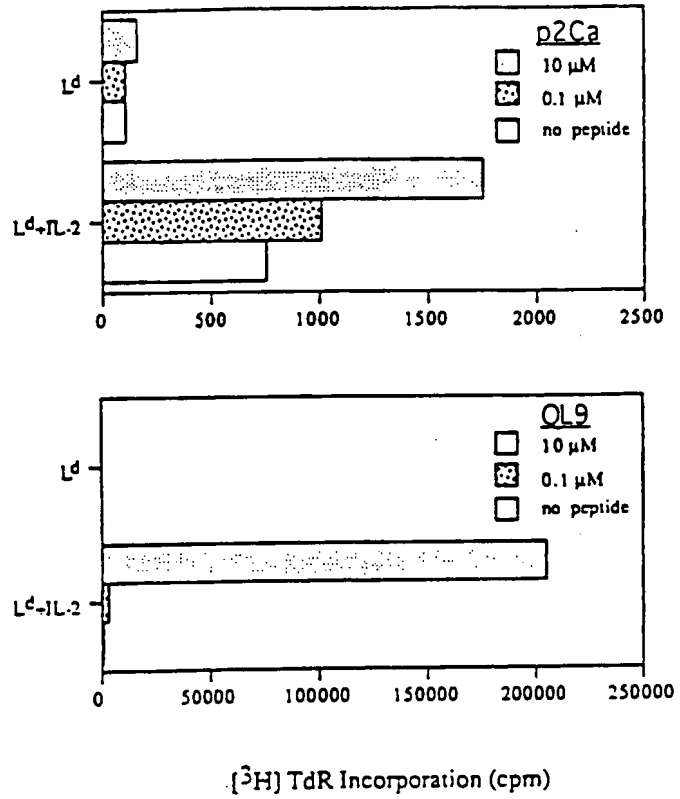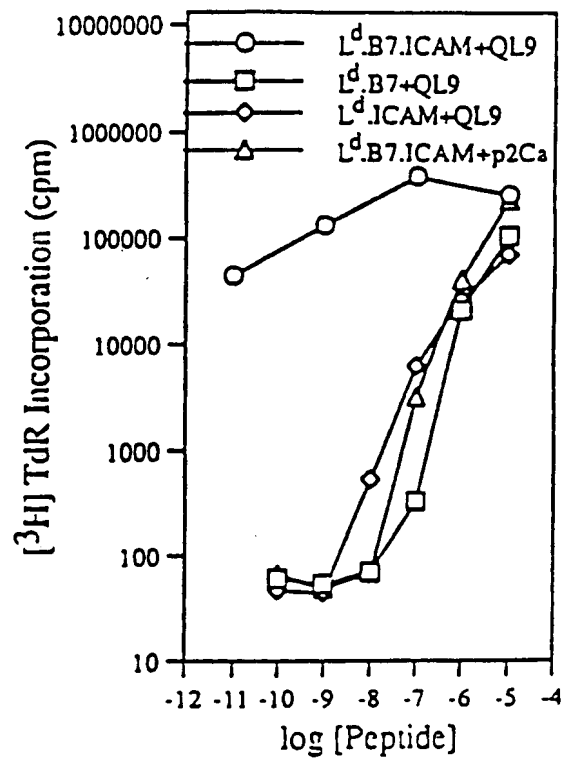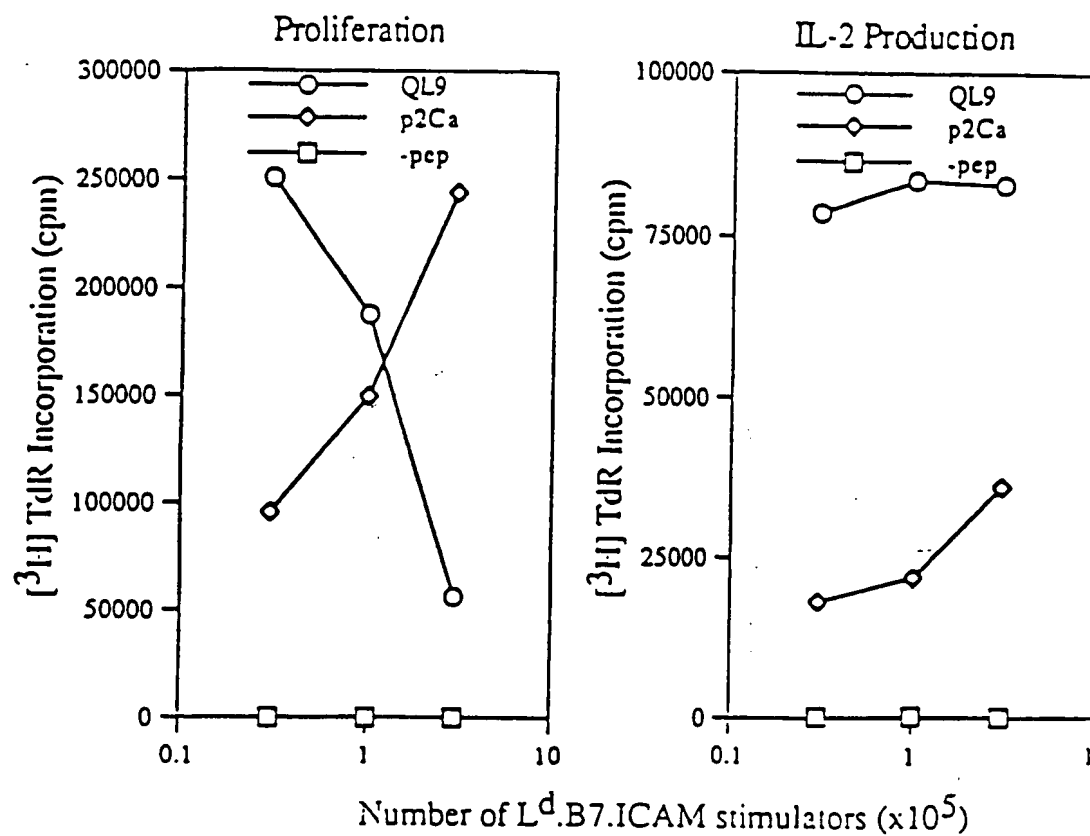
FIGURE 12

FIGURE 13

FIGURE 14
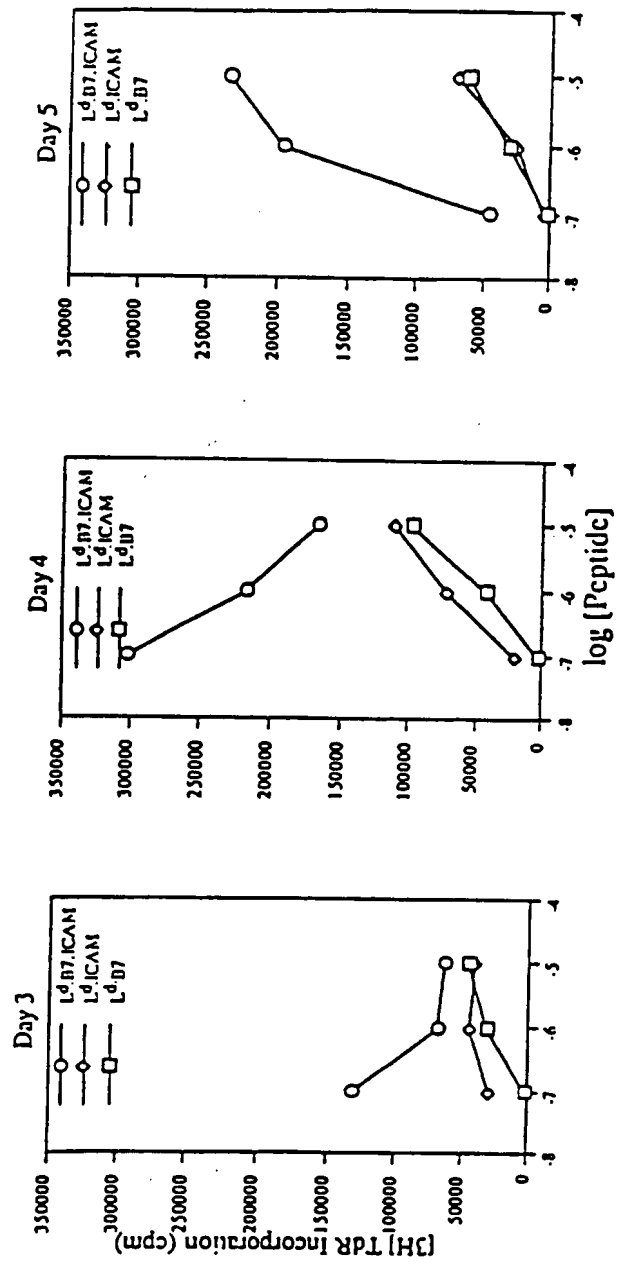
FIGURE 15
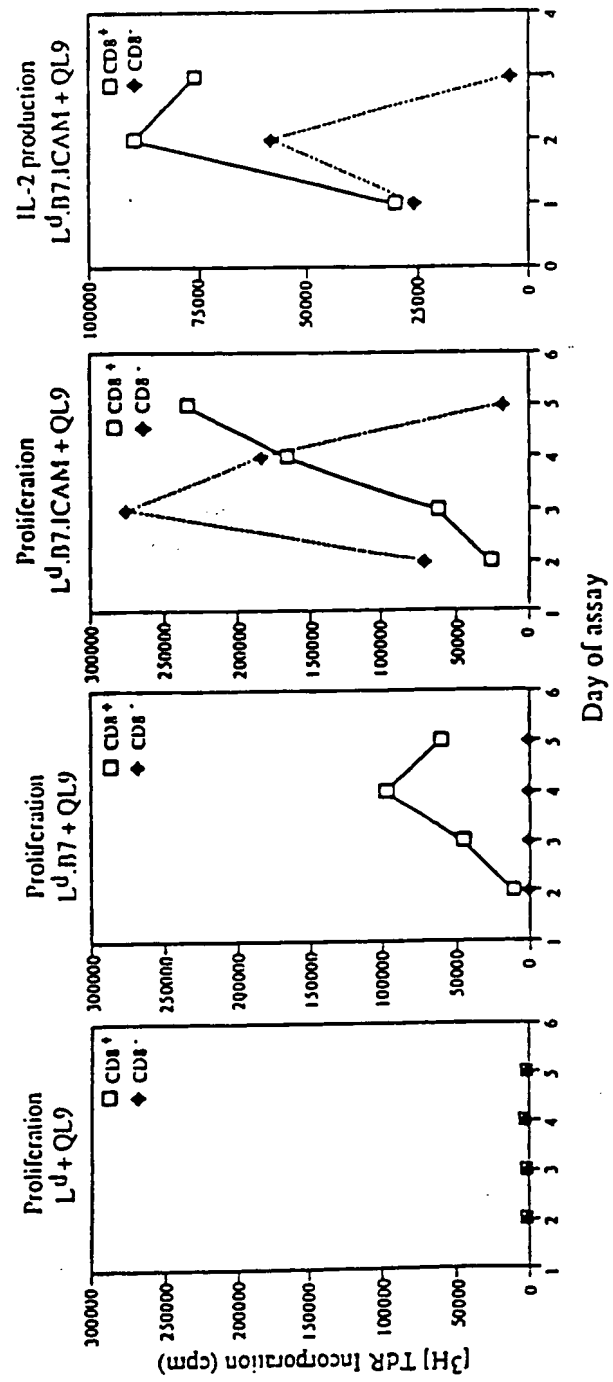
FIGURE 16
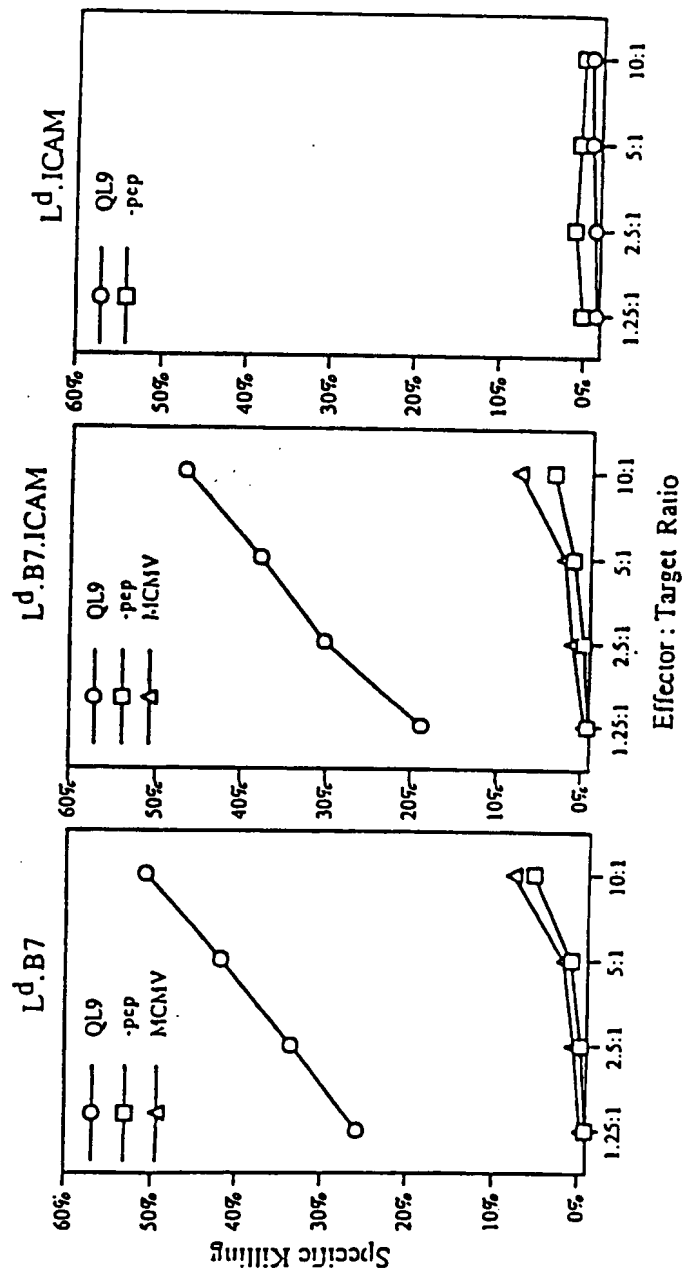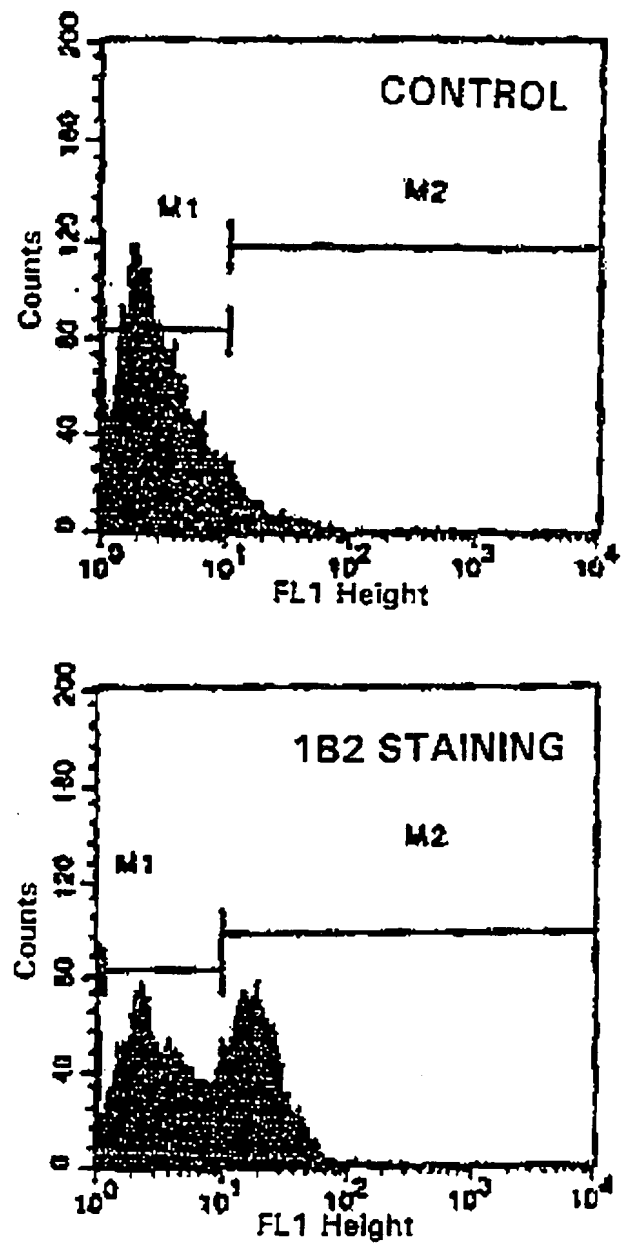
FIGURE 17
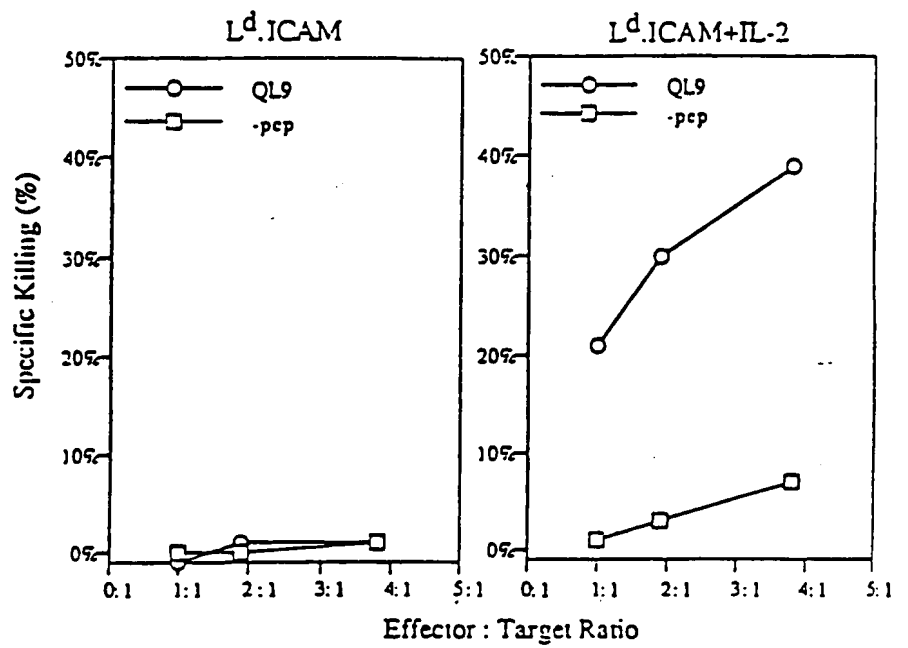
FIGURE 18
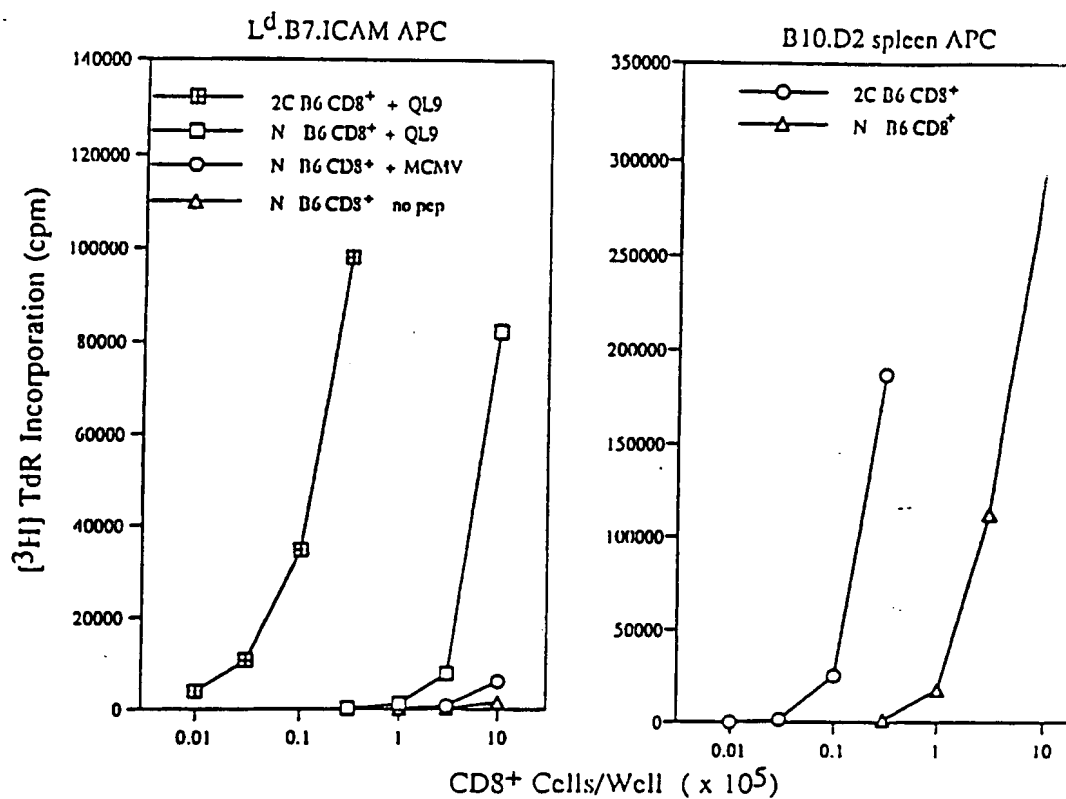
FIGURE 19
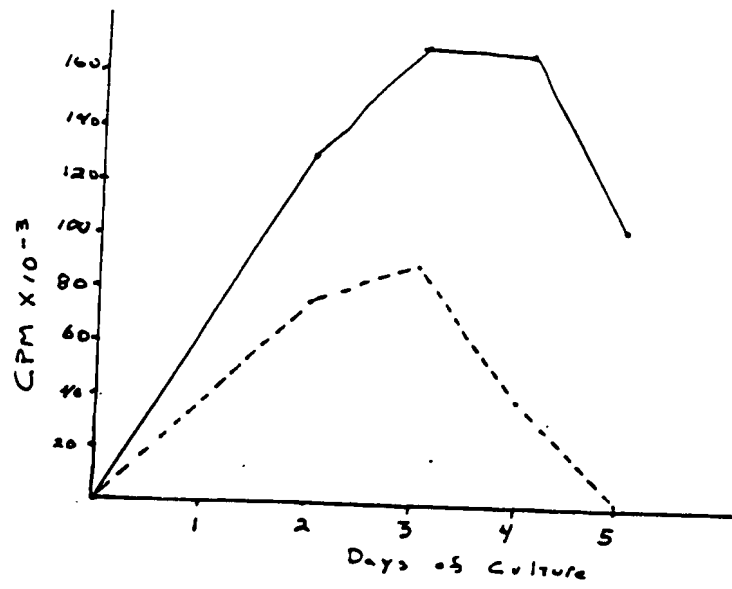
FIGURE 20
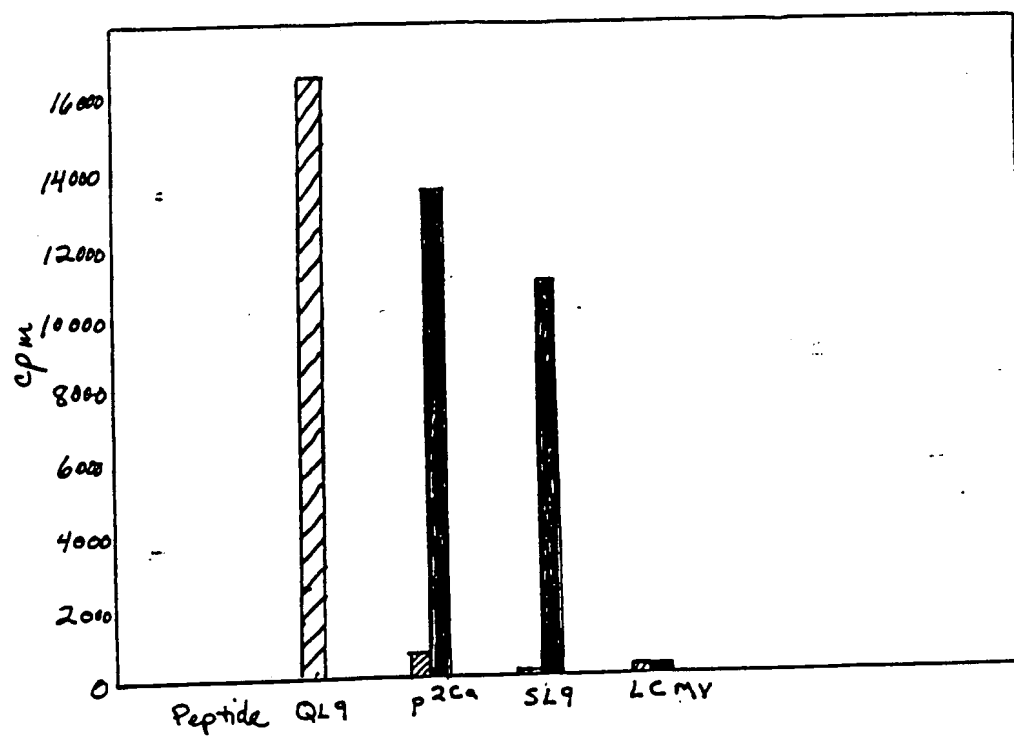
FIGURE 21
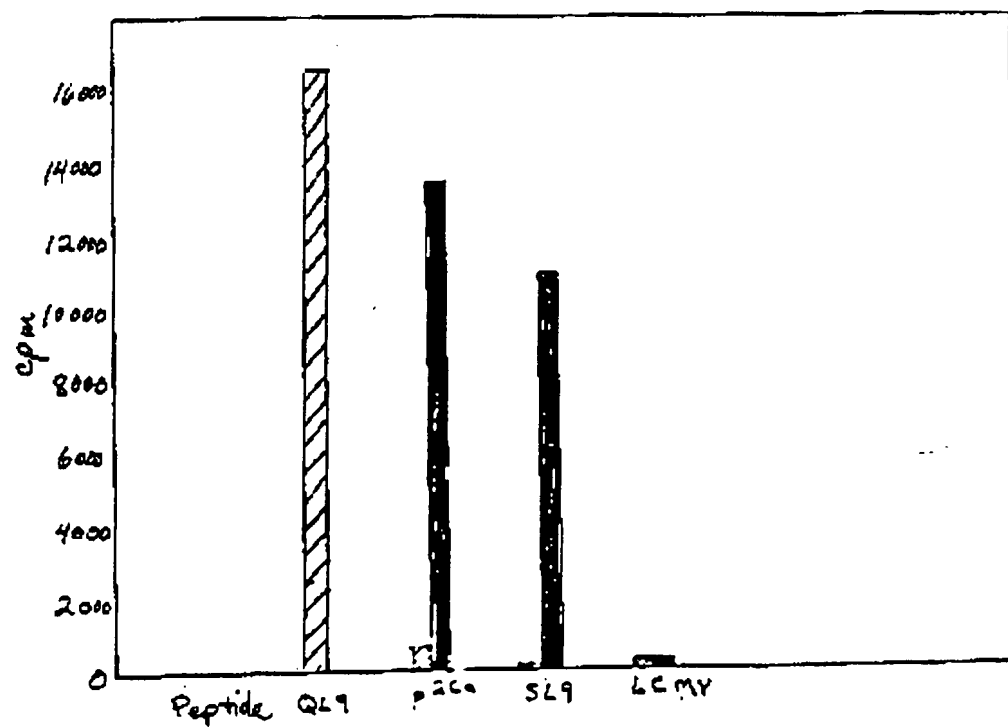
FIGURE 21

FIGURE 22
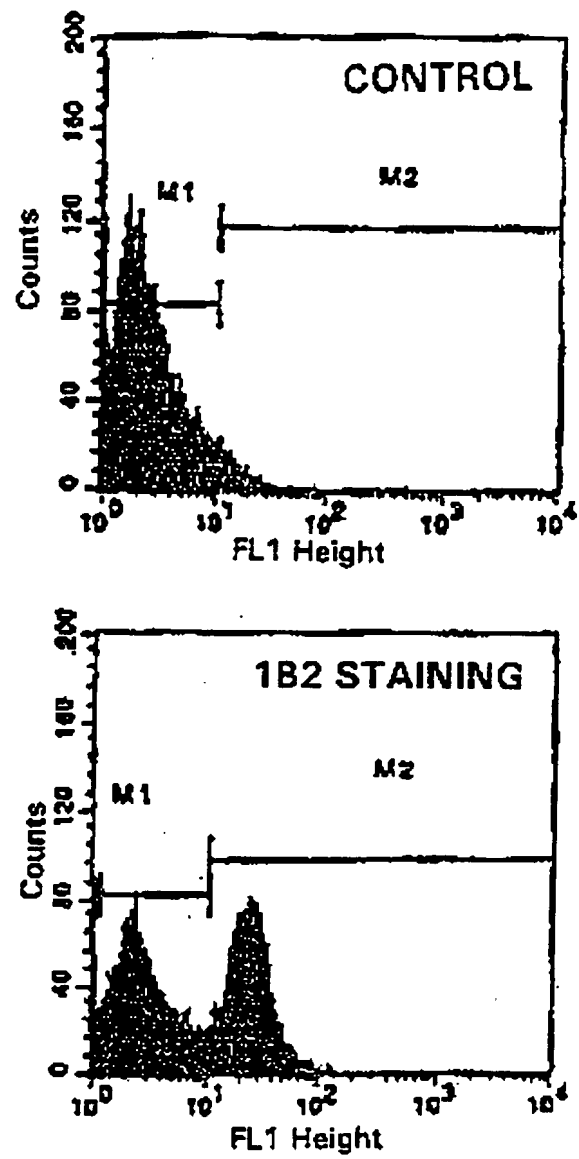
FIGURE 23
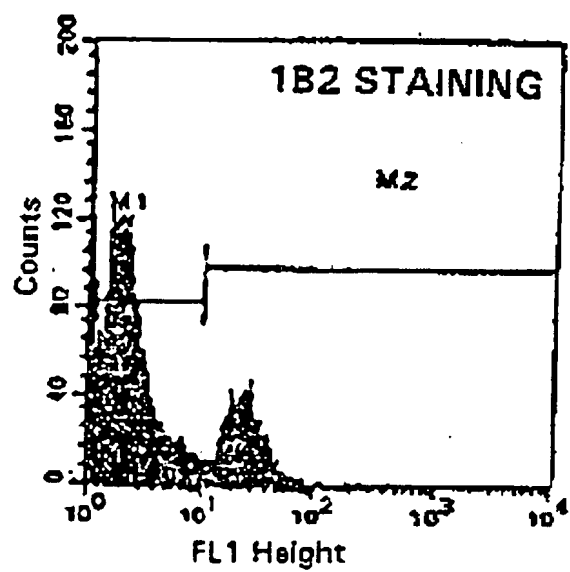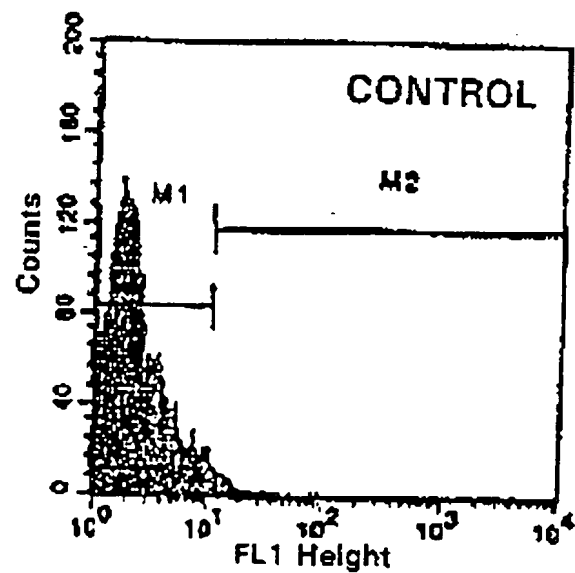
FIGURE 24
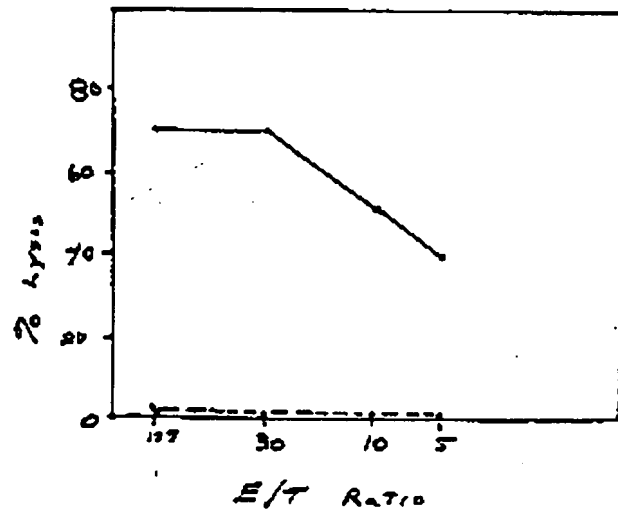
FIGURE 25

CTL Activity of Human CD8+ Cells Stimulated with Fly Cells
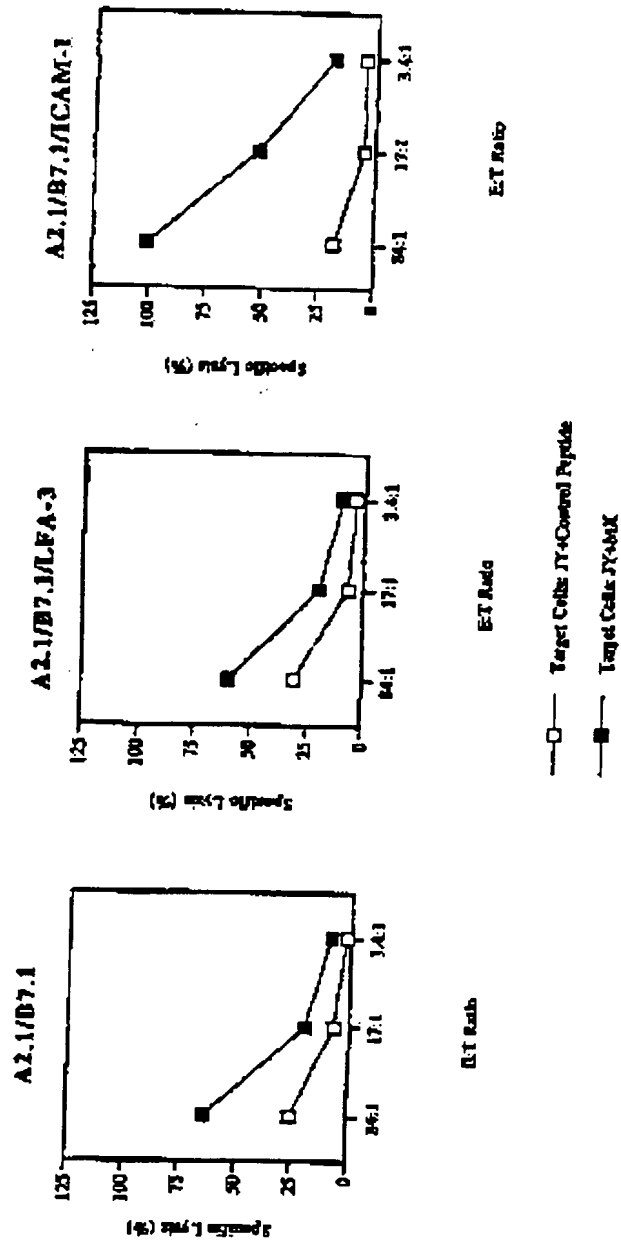Loaded with Influenza Matrix (MX) Peptide

FIGURE 26

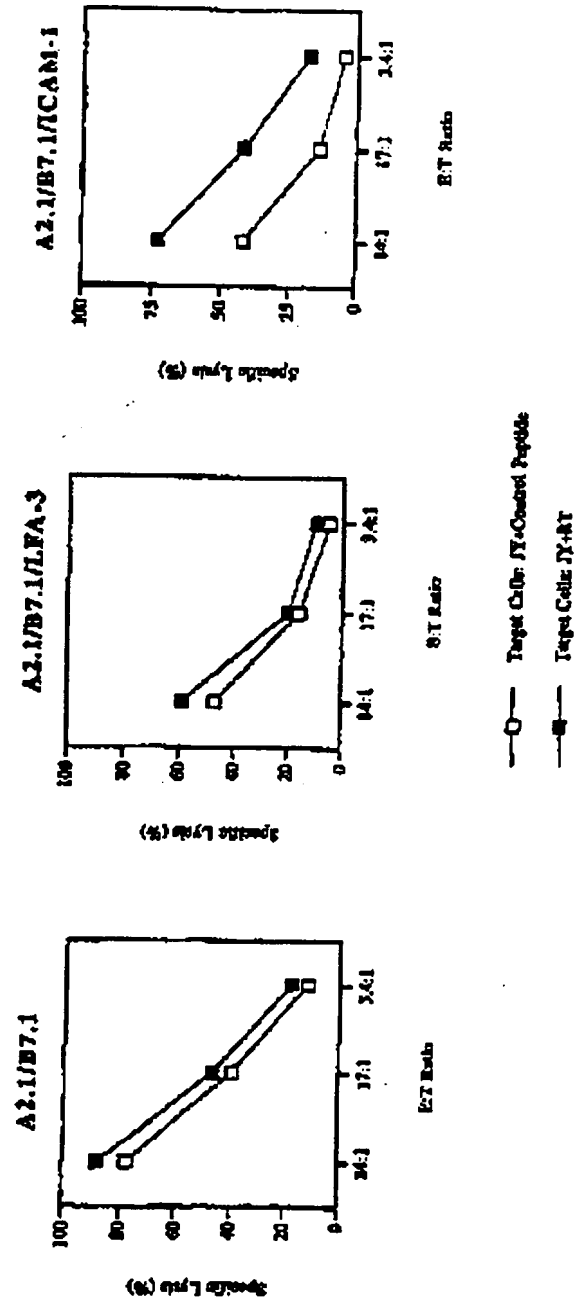CTL Activity of Human CD8+ Cells Stimulated with Fly Cells
Loaded with HIV-RT peptide

FIGURE 27

CTL Activity of Human CD8+ Cells Stimulated with Fly Cells
Loaded with Tyrosinase Peptide

FIGURE 28